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Document Processing Center (7407)
Office of Toxic Substances
U.S. Environmental Protection Agency
401 M Street, SW
Washington, DC 20460
Attn: TSCA Section 8(e) Coordinator

Dear Section 8(e) Docket Coordinator:

Re: TSCA 8(e) Supplemental Notice on Sulfonate-based Fluorochemicals

With this letter, 3M is providing final reports and other supplemental information related to previous TSCA Section 8(e) notifications. Many of the enclosed items are analytical reports providing blood serum and liver levels of test materials for which the in-life report referring to administered doses has already been submitted to the 8(e) docket. In other cases where the 8(e) notification consisted of preliminary data, we are submitting a final study report.

All of the enclosed items are already in EPA's possession and available in TSCA Docket AR-226. We believe, however, that placing these items in the 8(e) docket may allow for more convenient access to information directly related to previous 8(e) notifications by 3M.

The table below lists the enclosed items and references the study or data which already has been the subject of an 8(e) notification by 3M:

Attached Submission	Related Study/Data Already Filed Under 8(e)
1. Amended Analytical Study, 2(N-Ethylperfluorooctane sulfonamido)-ethanol in Two Generation Rat Reproduction, Determination of the Presence and Concentration of PFOS, M556, PFOSAA, and PFOSA in the Liver and PFOS, M556, PFOSAA, PFOSA and EtFOSE-OH in the Sera of Crl:CDBR VAF/Plus Rats Exposed to EtFOSE-OH, 3M Reference No. T-6316.5, Analytical Report TOX-013, LRN-U2095, June 11, 2001.	Combined Oral (Gavage) Fertility, Developmental and Perinatal/Postnatal Reproduction Toxicity Study of N-EtFOSE in Rats, 3M Reference No. T-6316.5, June 30, 1999, full report submitted February 15, 2000 to supplement earlier filing

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Attached Submission	Related Study/Data Already Filed Under 8(e)
2. Analytical Laboratory Report, Determination of the Presence and Concentration of Potassium Perfluorooctanesulfonate (CAS Number: 2759-39-3) in the Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage, Laboratory Report No. U2006, Requestor Project No. 3M TOX 6295.9, October 27, 1999. 3. Report Amendment 1, Combined Oral (Gavage) Fertility, Developmental and Perinatal/Postnatal Reproduction Toxicity Study of PFOS in Rats, Argus Research Laboratories, Inc., Protocol 418-008, Sponsor's Study No. 6295.9, April 13, 2000.	Combined Oral (Gavage) Fertility, Developmental and Perinatal/Postnatal Reproduction Toxicity Study of PFOS in Rats, Argus Research Laboratories, Inc., Sponsor's Study No. 6295.9, June 10, 1999, full report submitted February 15, 2000 supplementing earlier filing
4. Analytical Report, Determination of the Presence and Concentration of Perfluorooctanesulfonate, Perfluorooctanesulfonylamide, M556, and M570 in the Liver and Sera Samples, 3M Environmental Laboratory Ref. No. U2636, TOX-028, February 23, 2001	13-Week Dietary Study of N-Methyl Perfluorooctanesulfonamido Ethanol (N-MeFOSE) in Rats, 3M Ref. No. T-6314.1, Covance Study No. 6329-225, dated June 30, 2000, Section 8(e) filing July 24, 2000
5. Analytical Laboratory Report, Determination of the Concentration of PFOS, PFOSA, PFOSAA, and EtFOSE-OH in the Sera and Liver of Crl:CDBR VAF/Plus Rats Exposed to N-EtFOSE, 3M Environmental Laboratory Report No. TOX-098, Laboratory Request No. U2402, 3M Ref. No. T-6316.7, February 6, 2001.	Final Report, Oral (Gavage) Developmental Toxicity Study of 2(N-Ethylperfluorooctanesulfonamido)-ethanol in Rats, 3M Reference No. T-6316.7, December 17, 1998, submitted to Section 8(e) docket per letter of August 21, 2000
6. Analytical Laboratory Report on the Determination of the Presence and Concentration of Potassium Perfluorooctanesulfonate (PFOS) or another metabolite of 2(N-ethylperfluorooctanesulfonamido)-ethanol (N-EtFOSE) in Liver and Serum Specimens, 3M Environmental Laboratory Report No. TOX-097, Laboratory Request No. U2452, 3M Ref. No. T-6316.8, February 8, 2001	Final Report, Oral (Stomach Tube) Developmental Toxicity Study of N-EtFOSE in Rabbits, 3M Reference No. T-6316.8, January 11, 1999, submitted to Section 8(e) docket per letter of August 21, 2000
7. Final Report, Alexander, B., Mortality Studies of Workers Employed at the 3M Decatur Facility, University of Minnesota, April 26, 2001.	Preliminary data submitted to Section 8(e) docket in letter of December 15, 2000

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Attached Submission	Related Study/Data Already Filed Under 8(e)
8. Final Report, Acute Oral Toxicity Screen with T-3290CoC in Albino Rats, Safety Evaluation Laboratory, Riker Laboratories, Inc., Project No. 0882AR0362, 3M Reference No. T-3290 (40 % K^+PFOSAA in 3 % EtOH, 17 % IPA and 40 % H_20, L-6778, F-6873, Lot 501), November 5, 1982 *[Bibliography entry in Docket AR-226, final report was to be moved to TSCA 8(e) docket]*	Acute Oral Toxicity Screen with T-3290CoC in Albino Rats, Safety Evaluation Laboratory, Riker Laboratories, Inc., Project No. 0882AR0362, 3M Reference No. T-3290 (40 % K^+PFOSAA in 3 % EtOH, 17 % IPA and 40 % H_20, L-6778, F-6873, Lot 501), November 5, 1982, submitted to Section 8(e) docket in August 21, 2000 self-audit letter (which erroneously refers to rabbits rather than rats)
9. Giesy, J.P., and K. Kannan, Accumulation of Perfluorooctanesulfonate and Related Fluorochemicals in Fish Tissue, Michigan State University, June 20, 2001. 10. Giesy, J.P., and K. Kannan, Accumulation of Perfluorooctanesulfonate and Related Fluorochemicals in Mink and River Otters, Michigan State University, June 20, 2001. 11. Giesy, J.P., and K. Kannan, Perfluorooctanesulfonate and Related Fluorochemicals in Oyster, Crassostrea Virginica, From the Gulf of Mexico and Chesapeake Bay, Michigan State University, June 20, 2001. 12. Giesy, J.P. and K. Kannan, Perfluorooctanesulfonate and Related Fluorochemicals in Fish-Eating Water Birds, Michigan State University, June 20, 2001. 13. Giesy, J.P. and K. Kannan, Accumulation of Perfluorooctanesulfonate and Related Fluorochemicals in Marine Mammals, Michigan State University, June 20, 2001.	Preliminary data submitted to Section 8(e) docket May 26, 1999

If you have any questions about this submission, please contact me at (651)737-4795.

Sincerely,

Georjean Adams

Georjean Adams
Manager, 3M Corporate Product Responsibility

Enclosures

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MR 51614

ANALYTICAL LABORATORY REPORT

ON THE

Determination of the Presence and Concentration of Potassium Perfluorooctanesulfonate (CAS Number: 2759-39-3) in the Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

Laboratory Report No. <U2006>
Requester Project No. <3M TOX 6295.9>

Study Dates

Study Initiation: 26 May 1998
Study Completion: At Signature

8EHQ-80-373

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STUDY PERSONNEL AND CONTRIBUTORS

Analytical Chemistry Laboratories

3M Environmental Technology
and Safety Services
3M Environmental Laboratory
Fluorine Analytical Chemistry Team (FACT)
2-3E-09
935 Bush Avenue
St. Paul, MN 55106

Kris Hansen, Ph.D., *Analytical Study Director*
Lisa Clemen, *Analytical Chemist(s)*

In-life Testing Laboratory

Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, PA 19044

Raymond G. York, Ph.D., *Study Director*

Sponsor

3M Toxicology Services
3M Center
Building 220-2E-02
St. Paul, MN 55144

Marvin Case, *Sponsor Representative*

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STATEMENT OF COMPLIANCE

Study Title: Analytical Laboratory Report on Presence and Concentration of Potassium Perfluoro-octanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

Study Identification Number: FACT Tox-012

This study was conducted in compliance with Food and Drug Administration Good Laboratory Practice (GLP) Regulations for Nonclinical Laboratory Studies [Data Requirement(s): 21 CFR (Part 58)], with the exceptions in the bulleted list below. In addition, the present study has been audited retrospectively by an independent quality assurance unit. Audit procedures and findings for audits performed at the 3M Environmental Laboratory and at participating contract laboratories are housed with documentation pertinent to this study in archives at the 3M laboratory and will be retained for at least 10 years. The analytical portion completed at the 3M Environmental Lab was performed in accordance with 3M Environmental Technology and Safety Services Standard Operating Procedures.

Exceptions to GLP compliance:

- Storage containers for the reference substance, PFOS, were not labeled with name, CAS number, batch number, expiration date, or storage conditions.
- Details of the preparation, maximum storage time, and stability properties of the reference substance, PFOS, are unknown.
- The identity, strength, purity, and composition defining the test or control article was not determined at the time of the study; however, analyses to detail the characterization were ongoing at the time of this analytical study.
- Two separate study directors were assigned to the *in vivo* and the analytical portions of this study.
- From 15 September 1998 until 1 October 1998, the protocol was not sponsor approved and was during that time period not in compliance.
- Deviations were not consistently approved by the study director as required by GLP regulations.
- Data corrections were not always recorded as required by GLP regulations.
- Data were not always attributed to the individual responsible for recording the data.
- Records in electronic form do not meet the criteria set forth under 21 CFR(11).
- A finalized sample log-in /tracking system was not in place at the time of the study; however, draft documents did exist and were used to log in and track samples.

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- Not all raw data were verified by the study director.
- No in-phase audits were conducted during the present study.
- At the time of the present study, personnel files did not always contain documentation of equipment and method training; however, personnel files have been updated, and this noncompliance has been resolved.

Signature of Study Director: Kevin Hurn 27 Oct 99

Signature of Study Sponsor: Marvin T. Case 20 October 1999

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GLP STUDY
QUALITY ASSURANCE STATEMENT

Study Title: Analytical Laboratory Report on Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

Study ID Number: FACT Tox-012

This study has been inspected by the 3M Environmental Laboratory Quality Assurance Unit (QAU) as indicated in the following table. The findings were reported to the study director and management.

INSPECTION DATES		PHASE	DATE REPORTED TO	
From	*To*		*Management*	*Study Director*
9 August 1999	27 September 1999	Final Report	4 October 1999	4 October 1999

Kathleen M Stock 10-15-99

QAU Representative Date

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INTRODUCTION

Potassium Perfluorooctanesulfonate

CAS Number: 2795-39-3

Chemical Formula: $C_8F_{17}SO_3$-K+ Molecular Weight: 538

In the present study, groups of F0 rats were administered 0.1, 0.4, 1.6, or 3.2 mg PFOS kg/day in 0.5% Tween 80. (These doses correspond to concentrations of 0.02, 0.08, 0.32, and 0.64 mg/mL.) Groups of vehicle control F0 rats were administered only Tween 80. Male F0 animals were treated 42 days prior to mating and through the mating period; female F0 animals were administered PFOS daily 42 days prior to mating, through gestation, and up to 20 days following litter delivery. F1 male and female rats were exposed to the chemical *in utero* and during lactation. Following weaning at 21 days of age, selected F1 animals were treated during development and production of F2 animals. Various physical and biological parameters were monitored in F0, F1, and F2 animals.

In the analytical study reported here, liver and sera samples collected from the initial population of dosed animals (generation F0) and their offspring (generation F1) were analyzed for the presence of PFOS. (Analyses were performed to determine the presence of EtFOSE, PFOSA, POAA< PFOSEA, PFOSAA, and the monoester; however, these data were collected for informational purposes only, and were not reported.)

Liver samples were homogenized, and liver and sera samples were extracted by an ion-pairing extraction procedure. The extracts were quantitatively analyzed using high-pressure liquid chromatography/electrospray tandem mass spectrometry (HLPC/ESMSMS), and PFOS levels were evaluated against extracted standards. Analytical details are included in this report; further details are available in the study binder maintained by the 3M Fluor Analytical Chemistry Team (FACT).

Analyses assessing the toxicological effects of PFOS in the livers and sera of Sprague-Dawley rats were conducted in compliance with Good Laboratory Practice Regulations (21 CFR 58). Validated methods and standard operating procedures were followed during the preparation and analysis of the samples associated with this study.

SAMPLE RECEIPT

Tissue samples were received from Argus Research Laboratories sporadically, from August, 1998, through January, 1999. Samples received were packaged in dry ice. Specimens were registered with the 3M Environmental Laboratory and transferred to a freezer for storage. Sample receipt, identification, storage, and chain of custody protocols and data are located in the study folder for this report; the folder is located in the 3M archives.

Specimens analyzed at the 3M Environmental Laboratory will be maintained for a period of 10 years and will be stored at the laboratory at –20°C ± 10°C.

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MATERIALS AND METHODS

Chemical Characterization

Table 1 presents information regarding characterization of the test, control, and reference substances used in the analytical portion of this study.

	ANALYSES PERFORMED AT THE 3M ENVIRONMENTAL LABORATORY
Reference Substance	Potassium perfluorooctanesulfonate
Source	3M Specialty Chemical Division
Preparation	Details unknown
Maximum Storage Time	Unknown
Storage Conditions	In a sealed container at room temperature, exposed to light
Chemical Lot Number	193
Physical Description and Identity Analyses	White powder
Purity	99.28%
Stability	Unknown
Control Substance	Rat liver and serum matrices
Source	Rabbit liver—Argus Research Laboratories, Inc. Rabbit serum—Sigma Chemical Company
Preparation	Unknown
Maximum Storage Time	Unknown
Storage Conditions	Frozen at –20°C
Chemical Lot Number	Liver:F00012 Serum: 17H9306
Physical Description and Identity Analyses	Rabbit liver and serum
Purity	Unknown
Stability	Unknown

Table 1. Characterization and Treatment of the Control, Reference, and Test Materials in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

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	ANALYSES PERFORMED AT THE 3M ENVIRONMENTAL LABORATORY
Test Material	Rat serum and liver
Source	Sprague-Dawley rats exposed to PFOS via gavage during the *in vivo* portion of 3M TOX 6295.9 at Argus Research Laboratories
Preparation	Liver: extraction per FACT M-1.0 (refer to Attachment F) Serum: extraction per FACT M-3.0 (refer to Attachment F)
Maximum Storage Time	1 week
Storage Conditions	Frozen at –20°C
Chemical Lot Number	NA
Physical Description and Identity Analyses	Rat liver and serum
Purity	NA
Stability	NA

Table 1. Characterization and Treatment of the Control, Reference, and Test Materials in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage (continued)

Following analysis, remaining original specimens (test and control material) were stored frozen at –20°C and will be maintained for a period of 10 years.

Method Summaries

Following is a brief description of the methods used during this analytical study by the 3M Environmental Laboratory. Detailed descriptions of these methods are located in Attachment F.

Preparatory Methods:

- Method FACT-M-1.0: Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Fluorochemical Surfactants from Liver for analysis Using HPLC/ESMS.

  In this method, an ion pairing reagent was added to the sample and the analyte ion pair was partitioned into MtBE. Four mLs of the MtBE extract was transferred to a centrifuge tube and put onto a nitrogen evaporator until dry. Each extract was reconstituted in 1.0 mL of methanol, then filtered through a 3 cc plastic syringe attached to a 0.2 µm nylon filter into glass autovials.

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- Method FACT-M-3.0: Extraction of Potassium Perfluorooctanesulfonate or Other Fluorochemical Compounds from Serum or other Fluid for Analysis Using HPLC-Electrospray/Mass Spectrometry.

  Sera samples were extracted using an ion-pairing extraction procedure. In summary, an ion pairing reagent was added to the sample and the analyte ion pair was partitioned into methyl-*tert*-butyl ether (MtBE). Four mL of the MtBE extract was removed and put onto a nitrogen evaporator until dry. Each extract was reconstituted in 1.0 mL of methanol and filtered through a 3-cc plastic syringe attached to a 0.2 µm nylon filter into glass autovials.

Analytical Methods

- Method FACT-M-2.0: Analysis of Fluorochemicals in Liver Extract Using HPLC-Electrospray/Mass Spectrometry

  The analysis was performed by monitoring a single ion characteristic of a particular fluorochemical, such as the perfluorooctanesulfonate (PFOS) anion, m/z = 499. Additionally, samples were analyzed using a tandem mass spectrometer to further verify the identity of a compound by detecting daughter ions of the selected parent ion.

- Method FACT M-4.0: Analysis of Potassium Perfluorooctanesulfonate or other Fluorochemical in Serum or other Fluid Using HPLC-Electrospray/Mass Spectrometry

  The analysis was performed by monitoring a single ion characteristic of a particular fluorochemical, such as the perfluorooctanesulfonate (PFOS) anion, m/z= 499. Additionally, samples were analyzed using a tandem mass spectrometer to further verify the identity of a compound by detecting daughter ions of the parent ion.

Confirmatory dose analyses were conducted following the end of the study, and the results of these analyses are presented in Attachment G.

Analytical Equipment

Following are typical analytical equipment settings for the procedures used in the present study. These settings vary somewhat during actual data collection. Exact settings during all phases of data collection are recorded and presented in the analysis section of the study binder for FACT Tox-12.

HPLC System: Hewlett-Packard Series 1100 Liquid Chromatograph

Column:	Keystone Betasil C18 Column 2X100 mm, 5µm particle size
Flow rate:	300 µL/min
Solvent A:	2.0 mM ammonium acetate
Solvent B:	Methanol
Solvent Gradient:	40% to 90% B in 8.5 minutes Hold at 90% B for 3.0 minutes Return to 40% B in 1.0 minute Hold at 40% B for 1.0 minute
Injection Volume:	10 µL
Run Time:	13.5 minutes

Electrospray Tandem Mass Spectrometer: Micromass Quattro II API Mass Spectrometer

Mass Lynx 3.1 Software

Cone Voltage:	30—60V
Collision Gas Energy:	40 eV
Mode:	Electrospray Negative
Source Block Temperature:	115°C
Desolvation Temperature:	250°C
Electrode:	Z-spray
Primary Ion/Daughter Ions:	499/80, 99, 130, 180, 230 amu

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Protocol Deviations

There was one deviation to the protocol: The protocol called for the use of reference standard lot number 217; however, lot number 193 was used during the analytical portion of the study.

DATA SUMMARY, ANALYSES, AND RESULTS

Summary of Quality Control Analyses Results

- Calibration Check Standards: A mid-level, extracted matrix calibration check was analyzed at least every 10 samples to monitor instrument drift. Calibration check standards were compliant, and all calibration criteria were met (within ± 30%).
- Blanks: Four blanks were extracted concurrently with each batch of samples. Two extraction blanks were prepared with water as a surrogate matrix. Two additional samples of blank matrix were typically prepared with rabbit liver or sera and were used for extracted calibration curves. All blanks were nondetect for the compound(s) of interest.
- Duplicate matrix spike analyses including all target analytes were prepared and analyzed for one control animal. With a few exceptions, recoveries were within the acceptable range of 70-130%.

  Analysis of two liver matrix spike samples resulted in 51% and 61% recoveries of PFOS. These recoveries are below the lower boundary of the acceptable range; however, the second set of matrix spike samples were within the acceptable range. No action was taken to further characterize these matrix spikes.

  Analyses of both sets of sera matrix spikes resulted in PFOS recoveries greatly above the high limit of the acceptable range. Although 1 mL of sera was used for the analysis of PFOS levels in the sera of test animals, only a small amount of serum (500 µL) was available for the matrix spike preparation; this limited quantity of matrix may have been a source of error. As the extraction is scaled-down to accommodate the available matrix, the resulting extract is particularly susceptible to loss of solvent due to evaporation. These evaporative losses, which would not effect the PFOS concentration (PFOS has a low vapor pressure) in the extract, could result in a greater-than-expected PFOS concentration in the extract. When analyses of sera matrix spikes are performed with a full 1 mL of sera, high PFOS recoveries are unusual.
- Matrix Spikes: Matrix spikes and matrix spike duplicates were analyzed every 40 samples, with a minimum of two per batch during analytical analyses. The results of these analyses are located in the archived study binder.

Summary of Sample Results

- PFOS was detected in the livers of all control and exposed groups of F0 animals and F1 animals. PFOS was detected in the sera samples collected from all of the control and dosed F0 males and in sera samples collected from all dosed F0 females. The PFOS levels found in the samples from control animals were quite low—generally 100-less than levels detected in the low-dose group. PFOS concentration in exposed animals increased with increasing dose group, generally in a dose-related manner.

  PFOS levels in both sera and liver samples collected from female F0 animals were much less than levels detected in samples collected from the corresponding group of F0 males.

  PFOS concentrations measured in pooled liver samples collected from the exposed groups of F1 animals shortly after birth were roughly equal to or lower than concentrations measured in the corresponding groups of F0 females. No samples collected from F0 females that received 3.2 mg PFOS per kg/day were submitted for analysis. Table 1 summarizes results of the quantitative analyses performed on the F0 liver and sera samples examined.

Group	Dose (mg/kg/day)	Dose Concentration (mg/mL)	Average Concentration of PFOS in Serum (µg/mL)	Average Concentration of PFOS in Liver (µg/g)
0.0 mg/kg/day	0.0	0.0	Female: 0.0307 Male: 0.0244	Female: 0.171 Male: 0.665
0.1 mg/kg/day	0.1	0.02	Female: 5.28 Male: 10.5	Female: 14.8 Male: 84.9
0.4 mg/kg/day	0.4	0.08	Female: 18.9 Male: 45.4	Female: 58.0 Male: 176
1.6 mg/kg/day	1.6	0.32	Female: 82.0 Male: 152	Female: 184 Male: 323
3.2 mg/kg/day	3.2	0.64	Female: NR[a] Male: 273	Female: NR[a] Male: 1360

[a] Samples were not received.

Table 2. PFOS Concentrations in Serum and Liver Samples from F0 Male and Female Sprague-Dawley Rats

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PFOS concentrations measured in pooled liver samples collected from the **control and exposed** groups of F1 animals shortly after birth were roughly the equal to or lower than concentrations measured in the corresponding groups of F0 females. No samples collected from F1 males or females that received 3.2 mg/kg/day were submitted for analysis. PFOS was detected in the liver of all other exposed groups of F1 animals. Table 2 summarizes results of the quantitative analyses performed on the F1 sera samples examined.

Group	Dose (mg/kg/day)	Dose Concentration (mg/mL)	Average Concentration of PFOS in Liver (µg/g)
0.0 mg/kg/day	0.0	0.0	0.0511
0.1 mg/kg/day	0.1	0.020	6.19
0.4 mg/kg/day	0.4	0.080	57.6
1.6 mg/kg/day	1.6	0.320	70.4
3.2 mg/kg/day	3.2	0.640	NR[a]

[a] Samples were not received.

Table 3. PFOS Concentrations in Liver Samples from F1 Male and Female Sprague-Dawley Rats

Paper and electronic copies of raw data and study reports generated during the present studies will be kept for at least a period of time as established by regulation and by the 3M Standard Operating Procedures. Paper and electronic copies of reports, protocols, and methods generated by the 3M Environmental Technology and Safety Services laboratory will be retained with the study folder for at least a period of time as established by regulation and by the 3M Standard Operating Procedures.

Statistical Methods

For data generated by the 3M Environmental Laboratory, means and standard deviations were calculated using Microsoft Excel®, and relative standard deviations were calculated manually.

Standard deviation is a measurement of analytical precision; thus it is used to gauge the precision of the analyses. Relative standard deviation presents a measure of the magnitude of the standard deviation.

$$\sqrt{\frac{n\Sigma x^2-(\Sigma x)^2}{n(n-1)}}$$

Means are calculated by adding individual entities and dividing the resultant sum by the number of individual entities. Standard deviations were calculated using the equation:

Relative standard deviations were calculated by division of the standard deviation by the mean with subsequent multiplication of the quotient by 100.

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DATA QUALITY OBJECTIVES AND DATA INTEGRITY

No circumstances existed during the present study that would have affected the quality or integrity of the data. The following data quality objectives (DQOs) were followed during the study:

No circumstances existed during the present study that would have affected the quality or integrity of the data. The following data quality objectives (DQOs) were followed during the study:

- Linearity— The correlation coefficient (R^2) of the standard curve was equal to or greater than 0.98 using 1/x weighting
- Limits of detection—PFOS in serum=1.75 ppb
- Limits of quantitation—Equal to the lowest acceptable standard in the calibration curve
- Duplicate frequency/acceptable precision—<30%
- Spike frequency/acceptable recoveries—70% to 130%
- Use of confirmatory methods—no confirmatory methods were used
- Demonstration of specificity—specificity was demonstrated by chromatographic retention time and mass spectral daughter ion characterization

STATEMENT OF CONCLUSION

Statement of Conclusion:

Under the conditions of the present studies, potassium perfluorooctanesulfonate was observed in the livers and sera of all groups of rats exposed to the chemical during the *in vivo* portion of the studies.

REFERENCES

None

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ATTACHMENTS

- Attachment A: Report signature page
- Attachment B: Table of results of analysis of liver samples from F0 male and female Sprague-Dawley rats administered PFOS daily by gavage.
- Attachment C: Table of results of analysis of sera samples from F0 male and female Sprague-Dawley rats administered PFOS daily by gavage.
- Attachment D: Table of results of analysis of liver samples from F1 male and female Sprague-Dawley rats administered PFOS daily by gavage.
- Attachment E: In-life protocol and study protocol
- Attachment F: Preparatory and analytical methods
- Attachment G: Dose Analyses

ATTACHMENT A
REPORT SIGNATURE PAGE

Kristen J. Hansen, Ph.D., Study Director — Date: 10/27/99

Marvin T. Case, D.V.M., Ph.D., Study Sponsor — Date: 20 October 1999

Dale L. Bacon, Laboratory Director — Date: 10/27/99

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ATTACHMENT B

Dose Group	Sample Number	Extracted Weight (g)	PPOS Calculated Concentration (mg/g)	Total Amount of PFOS (µg/g)	Mean PFOS (µg/g)	Relative Standard Deviation
Method Blank	H_2O Blank 1	N/A	<MDL	<MDL		
Method Blank	H_2O Blank 2	N/A	<MDL	<MDL	<MDL	<MDL
Matrix Blank	Rabbit Liver (Blank 1)	N/A	<MDL	<MDL		
Matrix Blank	Rabbit Liver (Blank 2)	N/A	<MDL	<MDL	<MDL	<MDL
QC — 100 ppb	8801F-MS	1.0169	60	50%		
	8801F-MSD	1.0169	72	60%	55%	18%
	8801M-MS	1.0525	100	87%		
	8801M-MSD	1.0525	93	81%	84%	7%
0.0 mg/kg/day	8801F	1.0169	674	0.674		
	8807F	1.0384	158	0.158		10.1[a]
	8808F	1.0177	165	0.165	0.171[a]	0.0172[a]
	8821F	1.0439	197	0.197		82.9
	8833F	1.0048	166	0.166	0.272	0.225
	8101M	1.0525	910	0.910		
	8103M	1.0407	600	0.600		
	8104M	1.0568	577	0.577		
	8107M	1.0104	593	0.593		21.0
	8108M	1.0321	643	0.643	0.665	0.139
0.1 mg/kg/day	8838F	1.0374	17251	17.3		
(0.02 mg/mL)	8840F	1.0168	14433	144		
	8842F	1.0489	15557	15.6		
	8864F	1.0608	13807	13.8		11.6
	8870F	1.0225	12784	12.8	14.8	1.71
	8138M	1.0617	80270	80.3		
	8140M	1.0509	76629	76.6		
	8142M	0.9961	92106	92.1		
	8145M	1.036	87938	87.9		7.39
	8146M	0.9788	87451	87.5	84.9	6.28

Table 4. Analyses of Liver Samples from F0 Sprague-Dawley Rats in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

ATTACHMENT B (CONTINUED)

DOSE GROUP	SAMPLE NUMBER	EXTRACTED WEIGHT (g)	PPOS CALCULATED CONCENTRATION (mG/G)	TOTAL AMOUNT OF PFOS (µG/G)	MEAN PFOS (µG/G)	RELATIVE STANDARD DEVIATION
0.4 mg/kg/day	8890F	1.0346	61853	61.9		
(0.08 mg/mL)	8893F	1.0247	62547	62.5		
	8895F	1.0154	49543	49.5		
	8902F	1.0039	64081	64.1		11.6
	8905F	1.0426	51922	51.9	58.0	6.73
	8172M	1.0234	137715	138		
	8174M	1.0067	189256	189		
	8175M	1.0471	183050	183		
	8176M	1.0335	173288	173		13.3
	8181M	1.0197	198173	198	176	23.4
1.6 mg/kg/day	8919F	1.0756	200313	200		
(0.32 mg/mL)	8921F	1.0544	212684	213		
	8926F	1.0219	282001	282		
	8934F	1.022	183576	184		48.0
	8937F	1.654	40958	41.0	184	88.3
	8209M	1.0016	374773	375		
	8213M	1.0211	325087	325		
	8219M	1.0421	279320	279		
	8221M	1.0153	335349	335		11.2
	8225M	1.0316	301145	301	323	36.1
3.2 mg/kg/day	NR	NR	NR	NR		
(0.64 mg/mL)	NR	NR	NR	NR		
	NR	NR	NR	NR		
	NR	NR	NR	NR		NR
	NR	NR	NR	NR	NR	NR
	8243M	1.0065	1271156	1271		
	8244M	1.0397	1116251	1116		
	8249M	1.0107	2077414	207 7		
	8250M	1.0522	1168784	1169		30.0
	8255M	0.9908	1149853	1150	1360	40.7

* Sample 8801F value was excluded from the calculations.

Table 4. Analyses of Liver Samples from F0 Sprague-Dawley Rats in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage (continued)

ATTACHMENT C

DOSE GROUP	SAMPLE NUMBER	PFOS CALCULATED CONCENTRATION (ng/g)	TOTAL AMOUNT OF PFOS (µg/g)	MEAN PFOS (µg/g)	RELATIVE STANDARD DEVIATION (RSD)
Method Blank	H_2O Blank 1	<MDL			
Method Blank	H_2O Blank 2	<MDL	<MDL	<MDL	<MDL
Matrix Blank	Rabbit Liver (Blank 1)	<MDL	<MDL		
Matrix Blank	Rabbit Liver (Blank 2)	<MDL	<MDL	<MDL	<MDL
QC — 100 ppb	8801F-MS	60	50%		
	8801F-MSD	72	60%	55%	18%
	8801M-MS	100	84%		
	8801M-MSD	93	78%	81%	7%
0.0 mg/kg/day	8801F/M	369	0.369		
	8807F/M	51.5	0.0515		5.58[a]
	8808F/M	53.2	0.0532	0.0511[a]	0.00285[a]
	8821F/M	52.7	0.0527		124
	8833F/M	46.9	0.0469	0.115	0.142
0.1 mg/kg/day	8838F/M	5872	5.87		
(0.02 mg/mL)	8840F/M	5149	5.15		
	8842F/M	7390	7.39		
	8864F/M	6745	6.74		14.2
	8870F/M	5788	5.79	6.19	0.879
0.4 mg/kg/day	8890F/M	62050	62.1		
(0.08 mg/mL)	8893F/M	61745	61.7		
	8895F/M	47738	47.7		
	8902F/M	63057	63.1		11.7
	8905F/M	53492	53.5	57.6	6.72
1.6 mg/kg/day	8919F/M	78376	78.4		
(0.32 mg/mL)	8921F/M	58893	58.9		
	8926F/M	91956	92.0		
	8934F/M	65023	65.0		20.6
	8937F/M	57897	57.9	70.4	14.5

[a] Sample 8801F value not included in this calculation

Table 5. Analyses of Liver Samples from F1 Sprague-Dawley Rats in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

ATTACHMENT D

DOSE GROUP	SAMPLE NUMBER	PPOS REPORTED (mg/mL)	MEAN PFOS (µg/mL)	RELATIVE STANDARD DEVIATION (RSD)
Method Blank	H_2O Blank 1	<MDL		
Method Blank	H_2O Blank 2	<MDL	<MDL	<MDL
Matrix Blank	Rabbit Liver (Blank 1)	<MDL		
Matrix Blank	Rabbit Liver (Blank 2)	<MDL	<MDL	<MDL
QC — 100 ppb	8801F-MS	136%		
	8801F-MSD	133%	135%	1%
	8801M-MS	119%		
	8801M-MSD	121%	120%	1%
0.0 mg/kg/day	8801F	0.127		
	8807F	0.0229		30.9*
	8808F	0.0291	0.037*	0.00950*
	8821F	0.0445		87.6
	8833F	0.0265	0.0500	0.044
	8101M	0.0306		
	8103M	0.0311		
	8104M	0.0177		
	8107M	0.0213		2.48
	8108M	0.0214	0.0244	0.00605
0.1 mg/kg/day	8838F	5.14		
(0.02 mg/mL)	8840F	4.93		
	8842F	5.24		
	8864F	5.23		6.78
	8870F	5.89	5.28	0.358
	8138M	11.8		
	8140M	9.54		
	8142M	9.65		
	8145M	11.0		9.02
	8146M	10.4	10.5	0.946
0.4 mg/kg/day	8890F	20.9		
(0.08 mg/mL)	8893F	18.6		
	8895F	18.4		
	8902F	17.4		6.89
	8905F	19.1	18.9	1.30
	8172M	40.7		
	8174M	54.1		
	8175M	41.6		
	8176M	47.4		12.1
	8181M	43.3	45.4	5.49

Table 6. Analyses of Sera Samples from F0 Sprague-Dawley Rats in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage

Dose Group	Sample Number	PPOS Reported (mg/mL)	Mean PFOS (µg/mL)	Relative Standard Deviation (RSD)
1.6 mg/kg/day	8919F	75.3		
(0.32 mg/mL)	8921F	79.2		
	8926F	113		
	8934F	74.4		21.4
	8937F	68.4	82.0	17.5
	8209M	144		
	8213M	151		
	8219M	148		
	8221M	165		5.20
	8225M	153	152	7.91
3.2 mg/kg/day	NR	NR		
(0.64 mg/mL)	NR	NR		
	NR	NR		
	NR	NR		NR
	NR	NR	NR	NR
	8243M	249		
	8244M	242		
	8249M	257		
	8250M	361		18.3
	8255M	255	273	49.8

▪ Sample 8801F value not included in this calculation

Table 6. Analyses of Sera Samples from F0 Sprague-Dawley Rats in the Study of the Presence and Concentration of Potassium Perfluorooctanesulfonate in Serum and Liver of Sprague-Dawley® Rats Exposed to PFOS via Gavage (continued)

ATTACHMENT E
IN-LIFE PROTOCOL AND ANALYTICAL STUDY PROTOCOL

Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, Pennsylvania 19044
T: (215) 443-8710 F: (215) 443-8587

PROTOCOL 418-008

SPONSOR'S STUDY NUMBER: 6295.9

STUDY TITLE: Combined Oral (Gavage) Fertility, Developmental and Perinatal/Postnatal Reproduction Toxicity Study of PFOS in Rats.

PURPOSE: The purpose of this study is to test for toxic effects/ disturbances resulting from PFOS treatment of Crl:CD®BR VAF/Plus® male and female rats before cohabitation through mating, gestation and lactation. This study evaluates ICH Harmonised Tripartite Guideline stages A through F of the reproductive process and should detect effects on the estrous cycle, tubal transport, implantation, gestation, parturition, lactation and maternal behavior in female rats, on the development of the offspring of the treated male and female rats, and permit detection of functional effects (e.g., effects on libido or epididymal sperm maturation) that may not be detected by histological examinations of male rat reproductive organs. Because manifestations of effects induced during this period may be delayed in the offspring, observations will be continued through production of F2 generation litters.

TESTING FACILITY: Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, Pennsylvania 19044-1297
Telephone: (215) 443-8710
Telefax: (215) 443-8587

STUDY DIRECTOR: Raymond G. York, Ph.D., DABT
Associate Director of Research

SPONSOR: 3M Toxicology Services
3M Center, Building 220-2E-02
St. Paul, Minnesota 55144-1000

STUDY MONITOR: Marvin T. Case, D.V.M., Ph.D.
Telephone: (612) 733-5180
Telefax: (612) 733-1773

ALTERNATE STUDY MONITOR: Andrew M. Seacat, Ph.D.
Telephone: (612) 575-3161
Telefax: (612) 733-1773

REGULATORY CITATIONS:

Study Design as Modification of: U.S. Food and Drug Administration (1994). International Conference on Harmonisation; Guideline on detection of toxicity to reproduction for medicinal products. *Federal Register*, September 22, 1994, Vol. 59, No. 183.

U.S. Food and Drug Administration. Good Laboratory Practice Regulations; Final Rule. 21 CFR Part 58.

Japanese Ministry of Health and Welfare (1997). *Good Laboratory Practice Standard for Safety Studies on Drugs*, MHW Ordinance Number 21, March 26, 1997.

European Economic Community (1989). *Council decision on 28 July 1989 on the acceptance by the European Economic Community of an OECD decision/recommendation on compliance with principles of good laboratory practice*. Official Journal of the European Communities: Legislation. 32 (No. L 315; 28 October): 1-17.

REGULATORY COMPLIANCE:

This study will be conducted in compliance with the Good Laboratory Practice (GLP) regulations cited above.

All changes or revisions of this protocol shall be documented, signed by the Study Director and the Sponsor, dated and maintained with the protocol.

The Quality Assurance Unit (QAU) will audit the protocol, the raw data and the report, and will inspect critical phases of the study in accordance with the Standard Operating Procedures of Argus Research Laboratories, Inc.

The final report will include a statement signed by the Study Director that the report accurately reflects the raw data obtained during the performance of the study and that all applicable GLP regulations were followed in the conduct of the study. Should significant deviations from GLP regulations occur, each will be described in detail, together with how the deviation might affect the quality or integrity of the study.

STUDY SCHEDULE:

See ATTACHMENT 1 to the protocol.

TEST ARTICLE AND VEHICLE:

Identification:

Test Article:

Name:	PFOS.
Physical Description:	Light-colored powder.
Lot/Batch Number:	217.
Specific Gravity:	~0.6.
Purity:	98.9%.
Expiration Date:	May, 2000.

Information on the identity, composition, strength and purity of the test article is on file with the Sponsor.

Vehicle:

0.5% Tween 80 in Reversed Osmosis Membrane Processed Deionized Water (R.O. Deionized Water). Supplier and lot identification of Tween 80 to be documented in the raw data.

Neither the Sponsor nor the Study Director is aware of any potential contaminants likely to be present in the vehicle that would interfere with the results of this study. Therefore, no analyses other than those mentioned in this protocol will be conducted.

Safety Precautions:

Gloves, mask, appropriate eye protection and a uniform/lab coat are to be worn during formulation preparation and dosage administration. The Material Safety Data Sheet (MSDS) is attached to the protocol (ATTACHMENT 2).

Storage:

Bulk Test Article:	Room temperature.
Vehicle Components:	Room temperature.
Prepared Vehicle:	Room temperature.
Prepared Formulations:	Frozen (-20°C).

All test article shipments to the Testing Facility should be addressed to the attention of Julian Gulbinski, Manager of Formulations, at the previously cited address and telephone number.

Shipments should include information concerning storage conditions and shipping cartons should be labeled appropriately. The recipient should be notified in advance of shipment.

FORMULATION:

Frequency of Preparation:

Formulations (suspensions) will be prepared daily at the Testing Facility.

Detailed preparation procedures are attached to this protocol (ATTACHMENT 3).

Adjustment for Purity:

The test article will be considered 100% pure for the purpose of dosage calculations.

Testing Facility Reserve Samples:

The Sponsor will reserve a sample (1 g) of each lot of the bulk test article used during the course of this study. The Testing Facility will reserve a sample (5 mL) of each lot of the vehicle components used during the course of this study. Samples will be stored under the previously cited conditions.

ANALYSES:

Samples additional to those described below may be taken if deemed necessary during the course of the study.

Bulk Test Article Sampling:

No analyses of the bulk test article will be conducted during the course of this study. Information on the stability of the bulk test article is on file with the Sponsor.

Analyses of Prepared Formulations:

Stability:

Stability data for prepared formulations bracketing the range of concentrations and conditions of this study are on file with the Sponsor and will not be determined during the conduct of this study. Suspensions will be prepared daily at the Testing Facility.

Homogeneity Analyses:

Homogeneity of the test article in prepared suspensions will be verified during the course of this study. A syringe will be used to withdraw samples (5 mL each) from the top, middle and bottom of the highest concentration on the first day of preparation. Each sample (5 mL) will be divided into two aliquots, one of 2 mL and one of 3 mL. One aliquot (2 mL) will be shipped for analysis; the other aliquot (3 mL) will be retained at the Testing Facility as a backup sample. Backup samples will be stored under the previously cited conditions and discarded at the Testing Facility upon the request of the Sponsor.

Concentration Analyses:

Concentration of the prepared test article suspensions will be verified during the course of this study. A syringe will be used to withdraw samples (5 mL each) from each concentration during the first and sixth week of dosage administration. Each sample (5 mL each) will be divided into two aliquots, one of 2 mL and one of 3 mL. One aliquot (2 mL) will be shipped for analysis; the other aliquot (3 mL) will be retained at the Testing Facility as a backup sample. Backup samples will be stored under the previously cited conditions and discarded at the Testing Facility upon the request of the Sponsor.

Shipping Instructions:

Samples to be analyzed will be shipped (frozen on dry ice) to:

Kris J. Hansen, Ph.D.
3M Environmental Technology and Safety Services
935 Bush Avenue
Building 2-3E-09
St. Paul, Minnesota 55133-3331
Telephone: (612) 778-6018
Telefax: (612) 778-6176

Both the recipient and the Study Monitor will be notified in advance of sample shipment.

DISPOSITION:

Prepared formulations will be discarded at the Testing Facility. All remaining bulk test article will be returned to the Study Monitor at the previously cited address.

TEST SYSTEM:

Species/Strain and Reason for Selection:

The Crl:CD®BR VAF/Plus® (Sprague-Dawley) rat was selected as the Test System because: 1) this strain of rat has been demonstrated to be sensitive to reproductive and developmental toxins and has been widely used throughout industry for reproductive and developmental toxicity evaluations; 2) historical data and experience exist at the Testing Facility[1-3]; and 3) the test article is pharmacologically active in the species and strain.

Number:

Initial population acclimated: 195 virgin male and 205 virgin female rats.
Population selected for study: 175 male rats (35 per dosage group) and 175 female rats (35 per dosage group).

Ten mated female rats will be assigned to Caesarean-sectioning on day 10 of presumed gestation; the remaining female rats will be permitted to deliver litters.

250 F1 generation pups (25 per sex per dosage group) will be selected at weaning on day 21 postpartum for continued postnatal observation.

Body Weight and Age:

Male rats will be ordered to weigh from 300 g to 325 g each at receipt, at which time they will be expected to be at least 60 days of age. Female rats will be ordered to weigh from 200 g to 225 g each at receipt, at which time they will be expected to be at least 60 days of age. Actual body weights will be recorded the day after receipt and will be documented in the raw data. The weight ranges will be included in the final report.

Sex:

Both Fo and F1 generation male and female rats will be evaluated. Only Fo generation male and female rats will be given the test article.

Source:

Charles River Laboratories, Inc., Raleigh, North Carolina.

The rats will be shipped in filtered cartons by air freight and/or truck from Charles River Laboratories, Inc., to the Testing Facility.

Identification:

Fo Generation:

Rats are permanently identified using Monel® self-piercing ear tags (Gey Band and Tag Co., Inc., No. MSPT 20101). Male and female rats are assigned temporary numbers at receipt and given unique permanent identification numbers when assigned to the study before administration of the first dosage of the test article.

F1/F2 Generations:

Pups will not be individually identified during lactation; all parameters will be evaluated in terms of the litter. At weaning, each rat selected for continued observation will be identified with a Monel® self-piercing ear tag.

ANIMAL HUSBANDRY:

All cage sizes and housing conditions are in compliance with the *Guide for the Care and Use of Laboratory Animals*[4].

Housing:

Fo Generation Rats/F1 Generation Litters:

Fo generation rats will be individually housed in stainless steel wire-bottomed cages except during the cohabitation and postpartum periods. During cohabitation, each pair of rats will be housed in the male rat's cage. Beginning no later than day 20 of presumed gestation, Fo generation female rats assigned to natural delivery will be individually housed in nesting boxes. Each dam and delivered litter will be housed in a common nesting box during the postpartum period.

F1 Generation Rats/F2 Generation Litters:

After weaning, the F1 generation rats will be individually housed before cohabitation, housed in pairs (one male rat per female rat) during cohabitation, and individually housed after cohabitation. The same type of caging will be used as described for the Fo generation rats. Beginning no later than day 20 of presumed gestation, F1 generation female rats will be individually housed in nesting boxes. Each dam and delivered litter will be housed in a common nesting box during the postpartum period.

Nesting Material:

Bedding material (bed-o'cobs®) will be supplied to female rats assigned to natural delivery.

Bedding will be changed as often as necessary to keep the animals dry and clean. Analyses for possible contamination are conducted annually and documented in the raw data.

Room Air, Temperature and Humidity:

The animal room is independently supplied with at least ten changes per hour of 100% fresh air that has been passed through 99.97% HEPA filters (Airo Clean® room). Room temperature will be maintained at 64°F (18°C) to 79°F (26°C) and monitored constantly. Room humidity will also be monitored constantly and maintained at 30% to 70%.

Light:

An automatically controlled 12-hour light:12-hour dark fluorescent light cycle will be maintained. Each dark period will begin at 1900 hours EST.

Diet:

Rats will be given Certified Rodent Diet® #5002 (PMI Nutrition International) available *ad libitum* from individual feeders.

Water:

Water will be available *ad libitum* from individual bottles attached to the cages or from an automatic watering access system. All water will be from a local source and passed through a reverse osmosis membrane before use. Chlorine will be added to the processed water as a bacteriostat; processed water is expected to contain no more than 1.2 ppm chlorine at the time of analysis. Water is analyzed monthly for possible bacterial contamination and twice annually for possible chemical contamination.

Contaminants:

Neither the Sponsor nor the Study Director is aware of any potential contaminants likely to be present in the certified diet, the drinking water or the nesting material at levels that would interfere with the results of this study. Therefore, no analyses other than those routinely performed by the feed supplier or those mentioned in this protocol will be conducted.

RANDOMIZATION AND COHABITATION:

Fo Generation:

Upon arrival, rats will be assigned to individual housing on the basis of computer-generated random units. After acclimation, male and female rats will be selected for study on the basis of physical appearance and body weights recorded during acclimation. The rats will be assigned to dosage groups based on computer-generated (weight-ordered) randomization procedures.

Within each dosage group, consecutive order will be used to assign rats to cohabitation, one male rat per female rat. The cohabitation period will consist of a maximum of 14 days. Female rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ* will be considered to be at day 0 of presumed gestation and assigned to individual housing. Female rats not mated within the first 7 days of cohabitation will be assigned alternate male rats that have mated (same dosage group) and will remain in cohabitation for a maximum of seven additional days.

The first ten female rats per dosage group with a confirmed date of mating will be assigned to Caesarean-sectioning on day 10 of presumed gestation. The remaining female rats will be permitted to naturally deliver litters.

A table of random units will be used to assign five rats per group to a pharmacokinetic sample collection at scheduled sacrifice after completion of the cohabitation period (male rats siring litters with dams allowed to naturally deliver a litter) or on day 21 postpartum (female rats allowed to naturally deliver litters).

F1/F2 Generation Pups:

Day 1 of lactation (postpartum) is defined as the day of birth and is also the first day on which all pups in a litter are individually weighed (pup body weights will be recorded after all pups in a litter are delivered and groomed by the dam).

On day 4 postpartum, a table of random units will be used to select pups to be culled, and litters will be reduced to eight pups each. Whenever possible, the same number of male and female pups per litter will be continued on study.

At weaning of the F1 generation pups on day 21 postpartum, a table of random units will be used to select 25 male and 25 female pups per group, resulting in a total of 250 F1 generation rats (125 per sex) chosen for continued evaluation. At least one male pup and one female pup per litter, when possible, will be selected.

ADMINISTRATION:

Route and Reason for Choice:

The oral (gavage) route was selected for use because: 1) in comparison with the dietary route, the exact dosage can be accurately administered; and 2) it is one of the possible routes of human exposure.

Method and Frequency:

Dosages will be adjusted for the most recently recorded body weight and given at approximately the same time each day.

Fo Generation Male Rats:

Male rats will be given the test article once daily beginning 28 days before cohabitation (maximum 14 days) and continuing through the day before sacrifice. Male rats will be sacrificed after completion of the cohabitation period.

Fo Generation Female Rats:

Female rats will be given the test article once daily beginning 28 days before cohabitation (maximum of 14 days) and continuing through day 9 of presumed gestation (rats assigned to Caesarean-sectioning), day 24 of presumed gestation (rats assigned to natural delivery that do not deliver a litter) or day 20 postpartum (rats that deliver a litter).

F1 Generation:

F1 generation pups will not be directly given the test article, but may be possibly exposed to the test article during maternal gestation (*in utero* exposure) or via maternal milk during the lactation period.

Rationale for Dosage Selection:

Dosages will be selected by the Sponsor on the basis of previous studies conducted with the test article.

Dosage Levels, Concentrations and Volumes:

Dosage Group	Number of Rats Per Sex	Dosage (mg/kg/day)	Concentration (mg/mL)	Dosage Volume (mL/kg)	Argus Batch Number
I	35	0 (Vehicle)	0	5	B-418-008-A(Day.Month.Year)
II	35	0.1	0.02	5	B-418-008-B(Day.Month.Year)
III	35	0.4	0.08	5	B-418-008-C(Day.Month.Year)
IV	35	1.6	0.32	5	B-418-008-D(Day.Month.Year)
V	35	3.2	0.64	5	B-418-008-E(Day.Month.Year)

The test article will be considered 100% pure for the purpose of dosage calculations.

TESTS, ANALYSES AND MEASUREMENTS - Fo GENERATION:

Viability - Male and Female Rats:

All Periods: At least twice daily.

Clinical Observations and/or General Appearance - Male and Female Rats:

Acclimation Period: At least once.

Dosage Period: Twice daily. Prior to dosage administration and once approximately one hour postdosage.

Maternal Behavior: Days 1, 4, 7, 14 and 21 postpartum. Any observed abnormal behavior will be recorded daily.

Clinical observations may be recorded more frequently than cited above, if deemed appropriate by the Study Director and/or Study Monitor.

Body Weights - Male Rats:

Acclimation Period: At least once.

Dosage Period: Weekly.

Sacrifice: Terminal weight.

Body Weights - Female Rats:

Acclimation Period:	At least once.
Dosage Period:	Weekly to cohabitation. Daily during presumed gestation and on Days 1, 4, 7 and 14 postpartum (rats assigned to natural delivery).
Sacrifice:	Terminal weight.

Feed Consumption Values - Male Rats (recorded and tabulated):

Dosage Period:	Weekly.

Feed Consumption Values - Female Rats (recorded and tabulated):

Dosage Period:	Weekly to cohabitation. Daily during presumed gestation. Days 1, 4, 7 and 14 postpartum (rats assigned to natural delivery).

Feed consumption not tabulated after day 14 postpartum, when it is expected that pups will begin to consume maternal feed.

Feed Consumption Values - Male and Female Rats:

Feed consumption values may be recorded more frequently than cited above if it is necessary to replenish the feed. During cohabitation, when two rats occupy the same cage with one feed jar, replenishment of the feed jars will be documented. Individual values will not be recorded or tabulated.

Estrous Cycling and Mating:

A table of random units will be used to select 15 female rats per group for evaluation of estrous cycling by examination of vaginal cytology for 14 days before the start of the cohabitation period.

During cohabitation, all female rats will be evaluated daily until spermatozoa are observed in a smear of the vaginal contents and/or a copulatory plug is observed *in situ*.

Duration of Gestation:

The duration of gestation is calculated from day 0 of presumed gestation to the day the first pup is observed.

Fertility Parameters:

Fertility Index (percentage of matings that result in pregnancies).

Gestation Index (percentage of pregnancies that result in birth of live litters).

Number of offspring per litter (live and dead pups).

Number of implantation sites.

General condition of dam and litter during the postpartum period.

Viability Indices (percentage of pups born that survive 4 and 7 days).

Lactation Index (percentage of pups born that survive 21 days).

Caesarean-Sectioning Observations:

Rats will be Caesarean-sectioned on day 10 of presumed gestation. Placentae that appear abnormal (size, color or shape) will be noted in the raw data. The rats will be examined for number and distribution of:

Corpora Lutea.

Implantation Sites.

Viable and Nonviable Embryos.
(A viable embryo is oval or crescent shaped, pink, firm and enclosed in an amniotic sac filled with clear fluid. A nonviable embryo is amorphous, small, pale pink to tan or deep red to black, soft and enclosed in an amniotic sac filled with clear, cloudy, or opaque fluid.)

Natural Delivery:

Female rats will be evaluated for:

Clinical Observations During Parturition.

Duration of Gestation (day 0 of presumed gestation to the time the first pup is observed).

Length of Parturition (time of delivery of last pup minus the time of delivery of the first pup divided by N-1 pups in each litter).

Litter Size (defined as all pups delivered).

Pup Viability at Birth.

METHOD OF SACRIFICE - Fo GENERATION:

Rats will be sacrificed by carbon dioxide asphyxiation. Embryos will be discarded after examination.

NECROPSY - Fo GENERATION:

Gross lesions will be retained in neutral buffered 10% formalin for possible future evaluation (a table of random units will be used to select one control group rat of each sex from which all tissues examined at necropsy will be retained, in order to provide control tissues for any possible histopathological evaluations of gross lesions). Unless specifically cited below, all other tissues will be discarded.

Male and Female Rats Assigned to Pharmacokinetic Sample Collection:

At scheduled sacrifice after completion of the cohabitation period (male rats siring litters with dams allowed to naturally deliver a litter) and on day 21 postpartum (female rats allowed to naturally deliver a litter), five rats per group will be assigned to a pharmacokinetic sample collection. In addition to the appropriate evaluations described below, blood samples (approximately 4 mL per rat) will be collected from the inferior vena cava into serum separator tubes and centrifuged. The resulting serum (approximately 2 mL) will be immediately frozen on dry ice and maintained frozen (-70°C) until shipment to the Sponsor for analysis. The liver will be excised, weighed, and a sample section (lateral lobe) will be frozen and retained at -70°C until shipment to the Sponsor for analysis.

After completion of sample collection, serum and liver section (lateral lobe) samples will be shipped (frozen on dry ice) to Kris J. Hansen, Ph.D., at the previously cited address for analysis. Both the recipient and the Study Monitor will be notified in advance of sample shipment.

Scheduled Sacrifice of Male Rats:

After completion of the cohabitation period, male rats will be sacrificed and a gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. The following organs will be excised and weighed and retained for possible histologic evaluation: testes, epididymides, prostate and seminal vesicles (weighed with and without fluid). The testes will be fixed in Bouin's solution for 48 to 96 hours and then retained in neutral buffered 10% formalin for possible histopathological evaluation. The remaining organs will be retained in neutral buffered 10% formalin.

Scheduled Sacrifice - Female Rats Assigned to Caesarean-Sectioning:

On day 10 of presumed gestation, female rats will be sacrificed, Caesarean-sectioned, and a gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. Uteri of apparently nonpregnant rats will be stained with 10% ammonium sulfide to confirm the absence of implantation sites[5]. Uteri of nonpregnant rats and all ovaries will be retained in neutral buffered 10% formalin for possible future evaluation.

Scheduled Sacrifice - Female Rats Assigned to Natural Delivery:

Rats that do not deliver a litter will be sacrificed on day 25 of presumed gestation and examined for gross lesions. Uteri will be stained with 10% ammonium sulfide to confirm the absence of implantation sites[5].

After completion of the 21-day postpartum period, female rats will be sacrificed, and a gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. The number and distribution of implantation sites will be recorded.

Dams with No Surviving Pups:

Dams with no surviving pups will be sacrificed after the last pup is found dead, missing or presumed cannibalized. A gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. Postpartum data for these dams will be excluded from summary tables.

Rats Found Dead or Moribund:

Rats that die or are sacrificed because of moribund condition or abortion will be examined for the cause of death or moribund condition on the day the observation is made. The rats will be examined for gross lesions. Testes, epididymides, prostate and seminal vesicles of male rats will be excised and individual organ weights will be recorded (seminal vesicles weighed with and without fluid). The testes will be fixed in Bouin's solution for 48 to 96 hours and then retained in neutral buffered 10% formalin. The remaining organs will be retained in neutral buffered 10% formalin. Pregnancy status and uterine contents of female rats will be recorded. Aborted fetuses and/or delivered pups will be examined to the extent possible. Ovaries will be retained in neutral buffered 10% formalin. Uteri of apparently nonpregnant rats will be stained with 10% ammonium sulfide to confirm the absence of implantation sites[5].

TESTS, ANALYSES AND MEASUREMENTS - F1 GENERATION:

Viability:

Preweaning Period:	Litters will be observed for dead pups at least twice daily. The pups in each litter will be counted once daily.
Postweaning Period:	Twice daily.

Clinical Observations and/or General Appearance:

Preweaning Period:	Once daily.
Postweaning Period:	Once weekly.
Maternal Behavior:	Days 1, 4, 7, 14 and 21 postpartum. Any observed abnormal behavior will be recorded daily.

Clinical observations may be recorded more frequently than cited above, if deemed appropriate by the Study Director and/or the Study Monitor.

Body Weights:

Preweaning Period:	Days 1 (birth), 4, 7, 14 and 21 postpartum.
Postweaning Period:	Weekly.
Presumed Gestation Period:	Days 0, 7, 10, 14, 17 and 20 (female rats only).
Lactation Period:	Days 1, 4, 7, 10 and 14 (female rats only).
Sacrifice:	Terminal weight.

Feed Consumption Values (recorded and tabulated):

Preweaning Period:	Not recorded.
Postweaning Period:	Weekly except during cohabitation.
Presumed Gestation Period:	Days 0, 7, 10, 14, 17 and 20 (female rats only).
Lactation Period:	Days 1, 4, 7, 10 and 14 (female rats only).

Feed consumption values may be recorded more frequently if it is necessary to replenish the feed. During cohabitation, when two rats occupy the same cage with one feed jar, values will be documented when feed jars are filled. These intervals will not be tabulated.

Preweaning Developmental Observations:

The number of pups meeting the criterion is recorded on each day of testing. Testing continues until the day the criterion is attained by all pups in the litter.

Surface Righting Reflex (ability to right in 5 seconds): From day 1 postpartum.

Pinna Unfolding: From day 2 postpartum.

Eye Opening: From day 12 postpartum.

Acoustic Startle Response: From day 13 postpartum.

Air Righting Reflex: From day 14 postpartum.

Pupil constriction is evaluated once, on day 21 postpartum.

Postweaning Developmental Observations:

Sexual Maturation:

Female rats will be evaluated for the age of vaginal patency, beginning on day 28 postpartum. Male rats will be evaluated for the age of preputial separation, beginning on day 39 postpartum.

Passive Avoidance Testing:

Beginning at 24 ± 1 day postpartum, one male rat and one female rat from each litter, where possible, will be evaluated in a passive avoidance test for learning, short-term retention and long-term retention.

The passive avoidance apparatus consists of a two-compartment chamber with hinged Plexiglas® lids. One compartment is fitted with a bright light and Plexiglas® floor. The other compartment is fitted with a grid floor to which a brief (1 sec) pulse of mild electric current (1 mA) can be delivered. The two compartments are separated by a sliding door. On each test trial, the rat is placed into the "bright" compartment, the sliding door is opened and the light is turned on. The rat is allowed to explore the apparatus until it enters the "dark" compartment. The sliding door is then immediately closed, the light is turned off and the brief pulse of current is delivered to the grid floor. The rat is then removed from the apparatus and placed into a holding cage for 30 seconds before the start of the next trial. Trials are repeated until the rat remains in the "bright"

compartment for 60 seconds on two consecutive trials (the criterion for learning) or until 15 trials have been completed. The latency to enter the dark compartment or the maximum 60-second interval is recorded for each trial.

Each rat is tested twice. The test sessions are separated by a one-week interval, and the criterion is the same for both days of testing.

Dosage groups are compared for the following dependent measures:

The number of trials to the criterion in the first session--this measure will be used to compare groups for overall learning performance.

The latency (in seconds) to enter the "dark" compartment from the "bright" compartment on trial 1 in the first test session--this measure will be used to compare groups for activity levels and exploratory tendencies in a novel environment.

The latency (in seconds) to enter the "dark" compartment from the "bright" compartment on trial 2 in the first test session--this measure will be used to compare groups for short-term retention.

The number of trials to the criterion in the second test session--this measure will be used to compare groups for long-term retention.

The latency (in seconds) to enter the "dark" compartment from the "bright" compartment on trial 1 in the second session--this value is another indication of long-term retention.

Watermaze Testing:

Beginning at approximately 70 days postpartum, one male rat and one female rat from each litter will be evaluated in a water-filled M-maze for overt coordination, swimming ability, learning and memory.

Each rat is tested in a watertight 16-gauge stainless steel modified M-maze. The maze is filled with water to a depth of approximately nine inches, and the water is monitored for temperature (range of 21°C ± 1°C).

On each test trial, the rat will be placed into the starting position (base of the M-maze stem farthest from the two arms) and required to swim to one of the two goals of the M-maze, in order to be removed from the water. On the first trial, the rat is required to enter both arms of the maze before being removed from the water. The initial arm chosen on trial 1 is designated the incorrect goal during the remaining trials. Rats that fail to make a correct goal choice within 60 seconds in any given trial are guided to the correct goal and are then removed from the water. A 15-second intertrial interval will separate each trial. Each rat is required to reach a criterion of five consecutive errorless trials to terminate the test session. The maximum number of trials in any test

session is 15. Latency (measured in seconds) to choose the correct goal or the maximum 60-second interval is recorded for each trial, as is the number of errors (incorrect turns in the maze) during each trial.

Each rat is tested twice. The test sessions are separated by a one-week interval, and the correct goal and the criterion are the same for both test sessions.

Dosage groups are compared for the following dependent measures:

The number of trials to criterion on the first day of testing--this measure will be used to compare groups for overall learning performance.

The average number of errors (incorrect turns in the maze) for each trial on the first day of testing--this measure will also be used to compare groups for overall learning performance.

The latency (in seconds) to reach the correct goal on trial 2 of the first day of testing--this measure will be used to compare groups for short-term retention.

The number of trials to criterion on the second day of testing--this measure will be used to compare groups for long-term retention.

The average number of errors for each trial on the second day of testing--this measure will also be used to compare groups for long-term retention.

The latency (in seconds) to reach the correct goal on trial 1 of day 2 of testing--this is another indicator of long-term retention.

Reproductive Capacity:

At approximately 90 days of age, the F1 generation rats within each dosage group will be assigned to cohabitation, one male rat per female rat, based on computer-generated random units or random unit tables, with the exclusion of sibling matings. The cohabitation period will consist of a maximum of 14 days. Female rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ* will be considered to be at day 0 of presumed gestation and assigned to individual housing. Female rats that do not mate within the first 7 days of cohabitation will be assigned alternate male rats from the same dosage group that have mated. Female rats will be allowed to naturally deliver and maintain litters through a 21-day postpartum period.

Mating Performance:

As cited above for Fo generation rats.

Duration of Gestation:

As cited above for Fo generation rats.

Fertility Parameters:

As cited above for Fo generation rats.

F2 Generation Litter Data:

Viability, clinical observations and body weights for F2 generation pups will be recorded as cited above for F1 generation litters.

METHOD OF SACRIFICE - F1 GENERATION RATS/F2 GENERATION PUPS:

As previously cited for Fo generation rats.

NECROPSY - F1 GENERATION RATS:

Gross lesions will be retained in neutral buffered 10% formalin for possible future evaluation (a table of random units will be used to select one control group rat of each sex from which all tissues examined at necropsy will be retained, in order to provide control tissues for any possible histopathological evaluations of gross lesions). Unless specifically cited below, all other tissues will be discarded.

Scheduled Sacrifice - F1 Generation Male Rats:

Rats will be sacrificed after completion of the 14-day cohabitation period. A gross necropsy of the thoracic, abdominal and pelvic visceral will be performed. Testes and epididymides of male rats will be excised and individual organ weights will be recorded. The epididymides will be retained in neutral buffered 10% formalin. The testes will be fixed in Bouin's solution for 48 to 96 hours and then retained in neutral buffered 10% formalin.

Scheduled Sacrifice - F1 Generation Female Rats:

Female rats will be sacrificed after completion of the 21-day postpartum period. The number and distribution of implantation sites will be recorded. Rats that do not deliver a litter will be sacrificed on day 25 of presumed gestation and uteri will be stained with 10% ammonium sulfide to confirm the absence of implantation sites[5]. A gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. Female rats without a confirmed mating date that do not deliver a litter will be sacrificed on an estimated day 25 of presumed gestation.

F1 Generation Rats Found Dead or Moribund:

Rats that die or are sacrificed because of moribund condition or abortion will be examined for the cause of death or moribund condition on the day the observation is made. The rats will be examined for gross lesions. Testes, epididymides, prostate and seminal vesicles of male rats will be excised and individual organ weights will be recorded (seminal vesicles weighed with and without fluid). The testes will be fixed in Bouin's solution for 48 to 96 hours and then retained in neutral buffered 10% formalin. The remaining organs will be retained in neutral buffered 10% formalin. Pregnancy status and uterine contents of female rats will be recorded. Aborted fetuses and/or delivered pups will be examined to the extent possible. Ovaries will be retained in neutral buffered 10% formalin. Uteri of apparently nonpregnant rats will be stained with 10% ammonium sulfide to confirm the absence of implantation sites[5].

F1 Generation Dams with No Surviving Pups:

Dams with no surviving pups will be sacrificed after the last pup is found dead, missing or presumed cannibalized. A gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. Postpartum data for these dams will be excluded from summary tables.

F1/F2 Generation Pups Found Dead on Day 1 Postpartum:

Pups that die before examination of the litter for pup viability will be evaluated for vital status at birth. The lungs will be removed and immersed in water. Pups with lungs that sink will be identified as stillborn; pups with lungs that float will be identified as liveborn, and to have died shortly after birth. Pups with gross lesions will be preserved in Bouin's solution for possible future evaluation. Should postmortem autolysis preclude these evaluations, it will be noted in the necropsy data.

F1/F2 Generation Pups Found Dead or Moribund on Days 2 to 21 Postpartum:

Pups found dead or sacrificed due to moribund condition will be examined for gross lesions and for the cause of the moribund condition or death. Pups with gross lesions found on days 2 to 4 postpartum will be preserved in Bouin's solution for possible future evaluation; gross lesions of pups found on days 5 to 21 postpartum will be preserved in neutral buffered 10% formalin. Should postmortem autolysis preclude these evaluations it will be noted in the necropsy data.

F1/F2 Generation Pups Not Selected for Continued Observation:

F1 and F2 generation pups culled on day 4 postpartum will be sacrificed and examined for gross lesions; pups with gross lesions will be preserved in Bouin's solution. Necropsy will include a single cross-section of the head at the level of the frontal-parietal suture and examination of the cross-sectioned brain for apparent hydrocephaly.

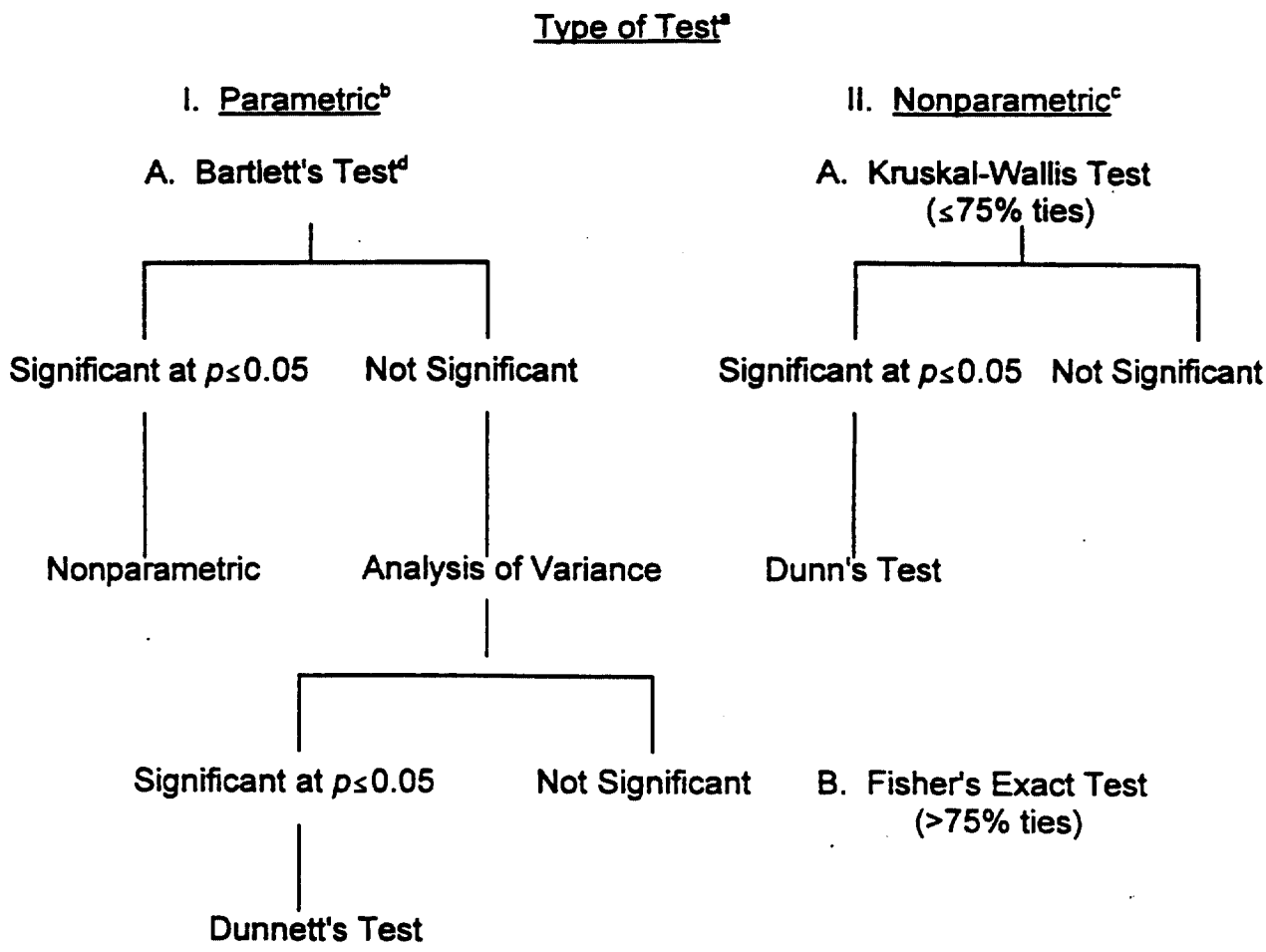
All F1 generation pups culled on day 21 postpartum will be sacrificed and examined for gross lesions; gross lesions will be preserved in neutral buffered 10% formalin. Necropsy will include a single cross-section of the head at the level of the frontal-parietal suture and examination of the cross-sectioned brain for apparent hydrocephaly.

Scheduled Sacrifice - F2 Generation Pups:

On day 21 postpartum, pups will be sacrificed and examined for gross lesions. Necropsy will include a single cross-section of the head at the level of the frontal-parietal suture and examination of the cross-sectioned brain for apparent hydrocephaly.

PROPOSED STATISTICAL METHODS[6-12]:

Averages and percentages will be calculated. Litter values will be used where appropriate. Additional procedures and/or analyses may be performed, if appropriate.



III. Test for Proportion Data

Variance Test for Homogeneity of the Binomial Distribution

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a. Statistically significant probabilities are reported as either $p \leq 0.05$ or $p \leq 0.01$.
b. Used only to analyze data with homogeneity of variance.
c. Proportion data are not included in this category.
d. Test for homogeneity of variance.

DATA ACQUISITION, VERIFICATION AND STORAGE:

Data will be hand- and/or computer-recorded. Records will be reviewed by the Study Director and/or appropriate management personnel within 21 days after generation. All original records will be stored in the archives of the Testing Facility. All original data will be bound and indexed. A copy of all raw data will be supplied to the Sponsor upon request. Preserved tissues will be stored at the Testing Facility at no charge for one year after mailing of the draft final report, after which time the Sponsor will be contacted to determine the disposition of these materials.

RECORDS TO BE MAINTAINED:

Protocol and Amendments.
Test Article, Vehicle and/or Reagent Receipt, Preparation and Use.
Animal Acquisition.
Randomization Schedules.
Mating History.
Treatment (if prescribed by Staff Veterinarian).
General Comments.
Clinical Observations and/or General Appearance.
Blood Sample Collection, Processing and Shipment.
Body Weights.
Feed Consumption Values.
Caesarean-Sectioning Observations.
Natural Delivery Observations.
Litter Observations.
Reflex and Physical Development and Behavioral Observations - F1 Generation Pups.
Gross Necropsy Observations.
Organ Weights (if required).
Photographs (if required).
Study Maintenance (room and environmental records).
Feed, Water and Bedding Analyses.
Packing and/or Shipment Lists.

KEY PERSONNEL:

Executive Director of Research: Mildred S. Christian, Ph.D., ATS
Director of Research: Alan M. Hoberman, Ph.D., DABT
Associate Director of Research and Study Director: Raymond G. York, Ph.D., DABT
Director of Laboratory Operations: John F. Barnett, B.S.
Manager of Study Coordination: Valerie A. Sharper, M.S.
Manager of Animal Operations and Member, Institutional Animal Care and Use Committee: Dena C. Lebo, V.M.D.
Manager of Regulatory Compliance: Kathleen A. Moran, M.S.
Consultant, Veterinary Pathology: W. Ray Brown, D.V.M., Ph.D., ACVP

FINAL REPORT:

A comprehensive draft final report will be prepared on completion of the study and will be finalized following consultation with the Sponsor. The report will include the following:

Summary and Conclusion.
Experimental Design and Method.
Evaluation of Test Results.
Appendices: Figures, Summary and Individual Tables Summarizing the Above Data, Protocol and Associated Amendments and Deviations, Study Director's GLP Compliance Statement, Reports of Supporting Data (if appropriate) and QAU Statement.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE STATEMENT:

The procedures described in this protocol have been reviewed by the Testing Facility's Institutional Animal Care and Use Committee. All procedures described in this protocol that involve study animals will be conducted in a manner to avoid or minimize discomfort, distress or pain to the animals.

The Sponsor's signature below documents the fact that information concerning the necessity for conducting this study and the fact that this is not an unnecessarily duplicative study may be obtained from the Sponsor. No alternative (*in vitro*) procedures were available for meeting the stated purposes of the study.

REFERENCES:

1. Christian, M.S. and Voytek, P.E. (1982). *In Vivo Reproductive and Mutagenicity Tests*. Environmental Protection Agency, Washington, D.C. National Technical Information Service, U.S. Department of Commerce, Springfield, VA 22161.

2. Christian, M.S. (1984). Reproductive toxicity and teratology evaluations of naltrexone (Proceedings of Naltrexone Symposium, New York Academy of Sciences, November 7, 1983), J. Clin. Psychiat. 45(9):7-10.

3. Lang, P.L. (1988). *Embryo and Fetal Developmental Toxicity (Teratology) Control Data in the Charles River Crl:CD®BR Rat*. Charles River Laboratories, Inc., Wilmington, MA 01887-0630. (Data base provided by Argus Research Laboratories, Inc.)

4. Institute of Laboratory Animal Resources (1996). *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, D.C.

5. Salewski, E. (1964). Färbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. Arch. Pathol. Exp. Pharmakol. 247:367.

6. Snedecor, G.W. and Cochran, W.G. (1967). Variance test for homogeneity of the binomial distribution. *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, pp. 240-241.

7. Sokal, R.R. and Rohlf, F.J. (1969). Bartlett's test of homogeneity of variances. *Biometry*, W.H. Freeman and Co., San Francisco, pp. 370-371.

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9. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. J. Amer. Stat. Assoc. 50:1096-1129.

10. Sokal, R.R. and Rohlf, F.J. (1969). Kruskal-Wallis Test. *Biometry*, W.H. Freeman and Co., San Francisco, pp. 388-389.

11. Dunn, O.J. (1964). Multiple comparisons using rank sums. Technometrics 6(3):241-252.

12. Siegel, S. (1956). *Nonparametric Statistics for the Behavioral Sciences*, McGraw-Hill, New York, pp. 96-104.

PROTOCOL APPROVAL:

FOR THE TESTING FACILITY

George E. Dearlove, Ph.D., DABT
Associate Director of Research

21-May-98
Date

Raymond G. York, Ph.D., DABT
Associate Director of Research
Study Director

21-MAY-98
Date

Barbara J. Patterson, B.A.
Chairperson, Institutional Animal Care and Use Committee

21-May-98
Date

FOR THE SPONSOR

Marvin T. Case, D.V.M., Ph.D.
Study Monitor

22 May 1998
Date

PRIMEDICA

Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, PA 19044
Telephone: (215) 443-8710
Telefax: (215) 443-8587

PROTOCOL 418-008

COMBINED ORAL (GAVAGE) FERTILITY, DEVELOPMENTAL AND PERINATAL/POSTNATAL REPRODUCTION TOXICITY STUDY OF PFOS IN RATS

SPONSOR'S STUDY NUMBER: 6295.9

Amendment 8 - 18 May 1999

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1. Concentration Analyses (page 5 of the protocol):

   The concentration samples were sent to the Sponsor. Sample analyses will be conducted at the discretion of the Sponsor and no report will be sent to the Testing Facility.

   Reason for Change:

   This change was made at the request of the Sponsor to clarify the protocol.

2. Male and Female Rats Assigned to Pharmacokinetic Sample Collection (page 14 of the protocol):

   The liver and serum samples were sent to the Sponsor. Samples will be analyzed at the discretion of the Sponsor.

   Reason for Change:

   This change was made at the request of the Sponsor to clarify the protocol.

3. F1/F2 Generation Pups Not Selected for Continued Observation (page 22 and Amendment 5 of the protocol):

   The stomach contents of these pups were sent to the Sponsor for analysis. Stomach contents will be analyzed at the discretion of the Sponsor.

Reason for Change:

This change was made at the request of the Sponsor to clarify the protocol.

George E. Dearlove 18-MAY-99

George E. Dearlove, Ph.D., DABT Date
Associate Director of Research

Raymond G. York 18-MAY-99

Raymond G. York, Ph.D., DABT Date
Associate Director of Research
Study Director

Theresa H. Worland 18 MAY 99

Dena C. Lebo, V.M.D. Date
Chairperson, Institutional Animal Care
and Use Committee

Marvin T Case 19 May 99

Marvin T. Case, D.V.M., Ph.D. Date
Study Monitor

3M ENVIRONMENTAL LABORATORY

PROTOCOL - ANALYTICAL STUDY
Potassium Perfluorooctanesulfonate in Two Generation Rat Reproduction

In-vivo study reference number: Argus 418-008
Study number: FACT 052798.1
Test substance: Potassium perfluorooctanesulfonate (PFOS)

Name and address of Sponsor:

Marvin Case
3M Toxicology Services
3M Center
Building 220-2E-02
St. Paul, MN 55144

Name and address of testing facility:

3M Environmental Technology and Services
935 Bush Avenue, Building 2-3E-09
St. Paul, MN 55106

Experimental start date:
Expected termination date: December 31, 1998
Method numbers and revisions:

FACT-M-1.0, Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Surfactants from Liver for Analysis Using HPLC-Electrospray/Mass Spectrometry

FACT-M-2.0, Analysis of Fluorochemicals in Liver Extracts Using HPLC-Electrospray/Mass Spectrometry

FACT-M-3.0, Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Surfactants from Serum for Analysis Using HPLC-Electrospray/Mass Spectrometry

FACT-M-4.0, Analysis of Fluorochemicals in Serum Extracts Using HPLC-Electrospray/Mass Spectrometry

Author: Lisa Clemen

Kris Hansen — 9/15/98
Kris Hansen — Date
Study Director

Marvin T Case — 10 Sept 1998
Marvin Case — Date
Sponsor Representative

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1.0 PURPOSE

The analytical portion of this dosing study is designed to evaluate levels of potassium perfluorooctanesulfonate (PFOS), or a metabolite of PFOS designated by the study director, in the liver of the parent and subsequent generations of the test system, or in the serum as necessary.

The in life portion of this study was conducted at Argus Research Laboratories.

2.0 REGULATORY COMPLIANCE

This study is conducted in compliance with the Food and Drug Administration Good Laboratory Practices regulation as stated in 21 CFR 58. Any exceptions will be noted in the final report.

3.0 TEST MATERIALS

3.1 Test, control, and reference substances and matrices

3.1.1 Analytical reference substance: Potassium perfluorooctanesulfonate (PFOS), lot # 217

3.1.2 Analytical reference substance matrix: Rat liver and serum

3.1.3 Analytical control substance: None

3.1.4 Analytical control substance matrix: Rat liver and serum

3.2 Source of materials

3.2.1 Analytical reference substance: 3M Specialty Chemical Division; traceability information will be included in the final report

3.2.2 Analytical reference substance matrix: Argus Research Laboratories; traceability information will be included in the final report

3.2.3 Analytical control matrix:

3.2.3.1 Rat liver – Argus Research Laboratories; traceability information will be included in the final report

3.2.3.2 Rat serum – Sigma Chemical Company; traceability information will be included in the final report

3.3 Number of test and control samples. Liver samples for testing were received from 40 test animals and 10 control animals. Serum samples will be tested at the discretion of the Study Director.

3.4 Identification of test and control samples: The samples are identified using the Argus Research Laboratories identifiers, which consist of a letter followed by the Argus project number, the animal number, the group designation, and the draw date.

3.5 Purity and strength of materials: Characterization of the purity and identity of the reference material is the responsibility of the Sponsor.

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3.6 **Stability of test material:** Characterization of the stability of the test material is the responsibility of the Sponsor.

3.7 **Storage conditions for test materials:** Test materials are stored at room temperature. Samples are stored at –20 ± 10 °C.

3.8 **Disposition of test and/or control substances:** Biological tissues and fluids are retained per GLP regulation.

3.9 **Safety precautions:** Refer to the material safety data sheets of chemicals used. Wear appropriate laboratory attire, and follow adequate precautions for handling biological materials and preparing samples for analysis.

4.0 EXPERIMENTAL - Overview

Tissues from animals dosed as described in Argus Research Laboratories Protocol #418-008 are received for analysis of fluorine compounds. At the discretion of the Study Director, a series of analytical tests will be performed on select tissues.

Initially, all liver samples will be analyzed for PFOS by electrospray/mass spectrometry (ES/MS). On the basis of findings from these analyses, additional sample matrices may be evaluated or other metabolites may be targeted. If additional analysis is performed, a protocol amendment will be written.

5.0 EXPERIMENTAL - Analytical Methods

5.1 **FACT-M-1.0,** Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Surfactants from Liver for Analysis Using HPLC-Electrospray/Mass Spectrometry

5.2 **FACT-M-2.0,** Analysis of Fluorochemicals in Liver Extracts Using HPLC-Electrospray/Mass Spectrometry

5.3 **FACT-M-3.0,** Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Surfactants from Serum for Analysis Using HPLC-Electrospray/Mass Spectrometry

5.4 **FACT-M-4.0,** Analysis of Fluorochemicals in Serum Extracts Using HPLC-Electrospray/Mass Spectrometry

6.0 DATA ANALYSIS

6.1 **Data transformations and analysis:** Data will be reported as the concentration (weight/weight) of PFOS per tissue or sample, or of PFOS per unit of tissue or fluid.

6.2 **Statistical analysis:** Statistics used may include regression analysis of the serum concentrations over time, and standard deviations calculated for the concentrations within each dose group. If necessary, simple statistical tests, such as Student's t test, may be applied to evaluate statistical difference.

7.0 Maintenance of Raw Data and Records

7.1 The following raw data and records will be retained in the study folder in the archives according to AMDT-S-8:

- **7.1.1** Approved protocol and amendments
- **7.1.2** Study correspondence
- **7.1.3** Shipping records
- **7.1.4** Raw data
- **7.1.5** Electronic copies of data

7.2 Supporting records to be retained separately from the study folder in the archives according to AMDT-S-8 will include at least the following:

- **7.2.1** Training records
- **7.2.2** Calibration records
- **7.2.3** Instrument maintenance logs
- **7.2.4** Standard Operating Procedures, Equipment Procedures, and Methods
- **7.2.5** Appropriate specimens.

8.0 References

8.1 3M Environmental Laboratory Quality System Chapters 1, 5 and 6

8.2 Other applicable 3M Environmental Laboratory Quality System Standard Operating Procedures

9.0 Attachments

9.1 **FACT-M-1.0,** Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Surfactants from Liver for Analysis Using HPLC-Electrospray/Mass Spectrometry

9.2 **FACT-M-2.0,** Analysis of Fluorochemicals in Liver Extracts Using HPLC-Electrospray/Mass Spectrometry

9.3 **FACT-M-3.0,** Extraction of Potassium Perfluorooctanesulfonate or Other Anionic Surfactants from Serum for Analysis Using HPLC-Electrospray/Mass Spectrometry

9.4 **FACT-M-4.0,** Analysis of Fluorochemicals in Serum Extracts Using HPLC-Electrospray/Mass Spectrometry

ATTACHMENT F:
PREPARATORY AND ANALYTICAL METHODS

3M ENVIRONMENTAL LABORATORY

METHOD

EXTRACTION OF POTASSIUM PERFLUOROOCTANESULFONATE OR OTHER ANIONIC FLUOROCHEMICAL SURFACTANTS FROM LIVER FOR ANALYSIS USING HPLC-ELECTROSPRAY/MASS SPECTROMETRY

Method Number: FACT-M-1.0

Adoption Date: 5/26/98

Revision Date: N/A

Author: Lisa Clemen

Approved By:

Laboratory Manager — Date: 5/26/98

Group Leader — Date: 5/26/98

Technical Reviewer — Date: 5/27/98

1.0 SCOPE AND APPLICATION

1.1 Scope: This method is for the extraction of Potassium Perfluorooctanesulfonate (PFOS) or other fluorochemical surfactants from liver.

1.2 Applicable Compounds: Fluorochemical surfactants or other fluorinated compounds.

1.3 Matrices: Rabbit, rat, bovine, and monkey livers or other livers as designated in the validation report.

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2.0 SUMMARY OF METHOD

2.1 This method describes how to extract potassium perfluorooctanesulfonate (PFOS) or other fluorochemical surfactants from liver using ion pairing reagent and 5.0 mLs of ethyl acetate. An ion pairing reagent is added to each sample and partitioned into ethyl acetate. Four mLs of extract is removed to a centrifuge tube and put onto a nitrogen evaporator until dry. Each extract is reconstituted in 1.0 mL methanol then filtered through a 3 cc plastic syringe attached to a 0.2 µm filter into glass autovials.

3.0 DEFINITIONS

3.1 None.

4.0 WARNINGS AND CAUTIONS

4.1 **Health and Safety Warnings:**

4.1.1 Use universal precautions when handling animal livers, they may contain pathogens.

5.0 INTERFERENCES

5.1 There are no known interferences at this time.

6.0 EQUIPMENT

6.1 The following equipment is used while carrying out this method. Equivalent equipment is acceptable.

6.1.1 Ultra-Turrax T25 Grinder for grinding liver samples
6.1.2 Vortex mixer, VWR, Vortex Genie 2
6.1.3 Centrifuge, Mistral 1000 or IEC
6.1.4 Shaker, Fberbach or VWR
6.1.5 Nitrogen Evaporator, Organomation
6.1.6 Balance

7.0 SUPPLIES AND MATERIALS

7.1 Gloves
7.2 Dissecting scalpels
7.3 Eppendorf or disposable pipettes
7.4 Nalgene bottles, capable of holding 250 mL and 1 L
7.5 Glass, type A, volumetric flasks
7.6 40 mL glass I-CHEM vials
7.7 Plastic sampule vials, Wheaton, 6 mL
7.8 Polypropylene centrifuge tubes, 15 mL
7.9 Labels

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7.10 Syringes, capable of measuring 10 µL to 50 µL

7.11 Glass, type A, volumetric pipettes

7.12 Graduated pipettes

7.13 Electronic pipettor, Eppendorf or equivalent

7.14 Timer

7.15 Disposable plastic 3 cc syringes

7.16 Filters, nylon syringe filters, 0.2 µm, 25 mm

7.17 Crimp cap autovials

Note: Prior to using glassware and bottles, rinse 3 times with methanol and 3 times with Milli-Q™ water. Rinse syringes a minimum of 9 times with methanol, 3 rinses from 3 separate vials.

8.0 Reagents and Standards

8.1 Reagents

8.1.1 Sodium Hydroxide (J.T Baker or equivalent), (NaOH) 10N: weigh approximately 200 grams NaOH. Pour into a 1000 mL beaker containing 500 liters (L) Milli-Q™ water, mix until all solids are dissolved. Store in a 1 L nalgene bottle.

8.1.2 Sodium Hydroxide (J.T Baker or equivalent), (NaOH) 1N. Dilute 10N 1:10. Measure 10 mL of the 10N NaOH solution into a 100 mL volumetric flask and dilute to volume using Milli-Q™ water. Store in a 125 mL nalgene bottle.

8.1.3 Tetrabutylammonium hydrogen sulfate (Kodak or equivalent), (TBA) 0.5M: Weigh approximately 169 grams of TBA into a 1 L volumetric containing 500 L Milli-Q™ water. Adjust to pH 10 using approximately 64 mL 10N NaOH and dilute to volume with Milli-Q™ water. Add NaOH slowly while adding the last 1 mL of NaOH because the pH changes abruptly. Store in a 1 L nalgene bottle.

8.1.3.1 TBA requires a check prior to each use to ensure pH = 10. Adjust as needed using 1N NaOH solution.

8.1.4 Sodium carbonate/Sodium Bicarbonate Buffer (J.T. Baker or equivalent), (Na_2CO_3/$NaHCO_3$) 0.25M: Weigh approximately 26.5 g of sodium carbonate (Na_2CO_3) and 21.0 g of sodium bicarbonate ($NaHCO_3$) into a 1 L volumetric flask and dilute to volume with Milli-Q™ water. Store in a 1 L nalgene bottle.

8.1.5 PFOS (3M Specialty Chemical Division), molecular weight = 538.

8.1.6 Ethyl Acetate, Omnisolv, glass distilled or HPLC grade.

8.1.7 Methanol, Omnisolv, glass distilled or HPLC grade.

8.1.8 Liver and control liver, received frozen from testing laboratory.

8.1.9 Milli-Q™ water, all water used in this method should be Milli-Q™ water and may be provided by a Milli-Q TOC Plus system.

8.2 Standards

8.2.1 Prepare PFOS standards for the standard curve.

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8.2.2 Weigh approximately 100 mg of PFOS into a 100 mL volumetric flask and record the actual weight.

8.2.3 Bring to volume with methanol for a stock standard of approximately 1000 ppm (µg/mL).

8.2.4 Dilute the stock solution with methanol for a working standard 1 solution of approximately 50 ppm.

8.2.5 Dilute the stock solution with methanol for a working standard 2 solution of approx. 5.0 ppm.

8.2.6 Dilute the stock solution with methanol for a working standard 3 solution of approx. 0.50 ppm.

9.0 Sample Handling

9.1 All livers are received frozen and must be kept frozen until the extraction is performed.

10.0 Quality Control

10.1 Matrix Spikes

10.1.1 Prepare and analyze matrix spike and matrix spike duplicate samples to determine the accuracy of the extraction.

10.1.2 Prepare each spike using liver chosen by the analyst, usually a control liver.

10.1.3 Expected concentrations will fall in the mid-range of the initial calibration curve.

10.2 Continuing Calibration Checks

10.2.1 Prepare and analyze continuing calibration check samples to determine the continued linearity of the initial calibration curve.

10.2.2 One check is prepared per group of ten samples. For example, if a sample set = 34, four checks are prepared and extracted.

10.2.3 Prepare each continuing calibration check from the same liver homogenate used to prep the initial curve.

10.2.4 The expected concentration will fall within the mid-range of the initial calibration curve.

11.0 Calibration and Standardization

11.1 Prepare Liver Homogenate to Use for Standards

11.1.1 Weigh approximately 40 g of liver into a 250 mL Nalgene bottle containing 200 mLs Milli-Q™ water. Grind to a homogeneous solution.

11.1.2 If 40 g is not available, use appropriate amounts of liver and water in keeping with a 1:5 ratio.

11.1.3 See section 13.0 to calculate the actual density of liver.

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11.1.4 Add 1 mL of homogeneous solution to a 15 mL centrifuge tube. Re-suspend homogeneous solution by shaking between aliquots while preparing a total of sixteen 1 mL aliquots of homogeneous solution in 15 mL centrifuge tubes.

11.1.5 Two 1 mL aliquots serve as matrix blanks. Use the standard concentrations and spiking amounts listed in table 1 to spike, in duplicate, two standard curves for a total of fourteen samples.

Table 1
Approximate Spiking Amounts for Calibration Standards

Working Standard (Approx. Conc.)	µL	Approx. final conc. of PFOS in liver
-	-	Blank
0.50 ppm	4	0.010 ppm
0.50 ppm	20	0.050 ppm
0.50 ppm	40	0.100 ppm
5.0 ppm	10	0.250 ppm
5.0 ppm	20	0.500 ppm
5.0 ppm	30	0.750 ppm
50 ppm	4	1.000 ppm

11.1.1 See section 13.0 to calculate actual concentrations of $\overline{\text{PFOS}}$ in calibration standards.

11.2 Extract spiked liver homogenates following **12.14-12.24** of this method. Use these standards to establish each initial curve on the mass spectrometer.

12.0 PROCEDURES

12.1 Obtain frozen liver samples. In spent tissue, note that the liver has not been packaged with other tissues.

12.2 Cut approximately 1 g of liver using a dissecting scalpel.

12.3 Weigh the sample directly into a tared plastic sampule vial.

12.4 Record the liver weight in the study notebook.

12.5 Label the sampule vial with the study number, weight, liver ID, date and analyst initials.

12.6 Add 2.5 mLs of water to sampule vial.

12.7 Grind the sample. Put the grinder probe in the sample and grind for about 2 minutes, or until the sample is homogeneous.

12.8 Rinse the probe into the sample with 2.5 mLs water using a pipette.

12.9 Take the grinder apart and clean it with methanol after each sample. Follow AMDT-EP-22.

12.10 Cap the sample and vortex for 15 seconds.

12.11 Pipette 1 mL homogenate into a 15 mL polypropylene centrifuge tube. Label the centrifuge tube with the identical information as the sampule vial. (See Worksheet for documenting the remaining steps.)

12.12 Spike liver homogenates with the appropriate amount of PFOS standard as described in section 11.1 or Table 1.

12.13 Pipette two 1 mL aliquots of Milli-Q™ water to centrifuge tubes. These will serve as instrument blanks.

12.14 Add 1 mL 0.5 M TBA and 2 mL of the 0.25 M sodium carbonate/sodium bicarbonate buffer.

12.15 Using a volumetric pipette, add 5 mLs ethyl acetate.

12.16 Cap each sample and put on the shaker for 20 minutes.

12.17 Centrifuge for 20 to 25 minutes, until layers are well separated. Set power on the centrifuge to approximately 3500 rpm.

12.18 Remove 4 mLs of organic layer, using a 5 mL graduated glass pipette, to a clean 15 mL centrifuge tube. Label this fresh tube with the same information as in **12.5**.

12.19 Put each sample on the analytical nitrogen evaporator until dry, approximately 2 to 3 hours.

12.20 Add 1.0 mL of methanol to each centrifuge tube using a graduated pipette.

12.21 Vortex mix for 30 seconds.

12.22 Attach a 0.2 µm nylon mesh filter to a 3 cc syringe and transfer the sample to this syringe. Filter into a 1.5 mL glass autovial.

12.23 Label the autovial with the study number, animal number and gender, sample timepoint, matrix, final solvent, extraction date, and analyst(s) who performed the extraction.

12.24 Cap and hold for electrospray mass spectrometry analysis.

12.25 Complete the worksheet and tape to page of study notebook.

13.0 Data Analysis and Calculations

13.1 Calculations:

13.1.1 Calculate the density of liver (mg) in 1.0 mL homogenate using the following equation:

$$\frac{\text{g of Liver} \times \text{Average weight of ten 1 mL aliquots (mg)}}{(\text{g of Liver} + \text{g of Water})}$$

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13.1.2 Calculate actual concentrations of PFOS in calibration standards using the following equation:

$$\frac{\mu L \text{ of Standard} \times \text{Concentration } (\mu g/mL)}{\text{mg Liver}^{*} / 1 \text{ mL homogenate}} = \text{Final Concentration } (\mu g/g \text{ or } mg/kg) \text{ of PFOS in Liver}$$

*Average weight of liver in solution as determined in **13.1.1**, by weighing ten 1 mL homogenates of approximately 40 mg liver in 200 mL of Milli-Q water.

14.0 Method Performance

14.1 The method detection limit is equal to half the lowest standard in the calibration curve.

15.0 Pollution Prevention and Waste Management

15.1 Sample waste is disposed in biohazard containers, flammable solvent waste is disposed in high BTU containers, and used glass pipette waste is disposed in broken glass containers located in the laboratory.

16.0 Records

16.1 Complete the extraction worksheet and tape into the study notebook.

17.0 Tables, Diagrams, Flowcharts, and Validation Data

17.1 The validation report associated with this method is FACT-M-1.0 & 2.0-V-1.

18.0 References

18.1 AMDT-EP-22, "Routine Maintenance of Ultra-Turrax T-25"

19.0 Affected Documents

19.1 FACT-M-2, "Analysis of Liver Extracts for Fluorochemicals using HPLC-Electrospray Mass Spectrometry"

20.0 Revisions

Revision Number.	Reason For Revision	Revision Date

Extraction Worksheet for FACT-M-1

Study #	Sample Number set #	PFOS approx. 0.5 ppm actual ppm #W_____	PFOS approx. 5 ppm actual ppm #W_____	PFOS approx. 50 ppm actual ppm #W_____	Date and Initials for Std.
-	H_2O Blank	-	-	-	
-	Liver Blank	-	-	-	
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[1] Study number where the original worksheet is located.

Blank	Liver Homogenate:	Std #	Liver amount = g
Liver Extraction Method		:	**Date & Initials**
Vortex 15 sec.			
Pipette 1 mL of Liver Solution			
Pipette 1 mL of †0.5 M TBA, pH 10.		Std. #_____	
Pipette 2 mL of 0.25 Na_2CO_3/0.25M $NaHCO_3$ Buffer		Std. #_____	
Pipette 5 mL of Ethyl Acetate		TN-A-_____	
Shake 20 min.			
Centrifuge 20-25 min.	Centrifuge	Speed	
Remove a 4 mL aliquot of organic layer			
Put on Nitrogen Evaporator to dryness	Evaporator	Temperature	
Add 1.0 mL of Methanol		TN-A-_____	
Vortex 30 sec.			
Filter using a 3cc B-D syringe with a 0.2µm SRI filter into a 1.5 mL autosample vial			

MS/MSD/___ Cont. Checks: Spiked _____ uL of a _____ ppm std (_____) for a final concentration of _____ ppm. MS/MSD used sample _____. Cont. Checks used same homogenate as for std curve.

3M ENVIRONMENTAL LABORATORY

METHOD

ANALYSIS OF FLUOROCHEMICALS IN LIVER EXTRACTS USING HPLC-ELECTROSPRAY/MASS SPECTROMETRY

Method Number: FACT-M-2.0

Adoption Date: 5/26/98

Revision Date: N/A

Author: Lisa Clemen

Approved By:

Laboratory Manager — Date: 5/26/98

Group Leader — Date: 5/26/98

Technical Reviewer — Date: 5/27/98

1.0 SCOPE AND APPLICATION

1.1 Scope: This method is for the analysis of extracts of liver or other tissues for fluorochemical surfactants using HPLC-electrospray/mass spectrometry.

1.2 Applicable Compounds: Potassium perfluorooctanesulfonate, anionic fluorochemical surfactants, or other ionizable compounds.

1.3 Matrices: Rabbit, rat, bovine, and monkey livers or other livers as designated in the validation report.

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2.0 SUMMARY OF METHOD

2.1 This method describes the analysis of fluorochemical surfactants extracted from liver using HPLC-electrospray/mass spectrometry. The analysis is performed by monitoring a single ion characteristic of a particular fluorochemical, such as the potassium perfluorooctanesulfonate (PFOS) anion, M/Z= 499. Samples may also be screened to verify compound identification.

3.0 DEFINITIONS

3.1 None.

4.0 WARNINGS AND CAUTIONS

4.1 Health and Safety Warnings:

4.1.1 Use caution with the voltage cable for the probe. When the voltage cable is plugged into the probe DO NOT TOUCH THE PROBE, there is risk of electrical shock.

4.2 Cautions:

4.2.1 Do not run solvent pumps above capacity of 400 bar (5800 psi). If pressure goes over 400 bar, the HP1100 will initiate automatic shutdown.

4.2.2 Do not run solvent pumps to dryness.

5.0 INTERFERENCES

5.1 Teflon should not be used for sample storage or any part of instrumentation that comes in contact with the sample or extract.

6.0 EQUIPMENT

6.1 Equipment listed below may be changed in order to optimize the system.

6.1.1 Micromass Electrospray Mass Spectrometer

6.1.2 HP1100 low pulse solvent pumping system and autosampler.

7.0 SUPPLIES AND MATERIALS

7.1 Supplies

7.1.1 Nitrogen gas, refrigerated liquid, regulated to approximately 100 psi.

7.1.2 HPLC column, specifics to be determined by the analyst.

7.1.3 Capped autovials or capped 15 mL centrifuge tubes.

8.0 REAGENTS AND STANDARDS

8.1 Reagents

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8.1.1 Methanol, HPLC grade or equivalent.

8.1.2 Milli-Q™ water, all water used in this method should be Milli-Q™ water and may be provided by a Milli-Q TOC Plus system.

8.1.3 Ammonium acetate, HPLC grade or equivalent.

8.2 Standards

8.2.1 Typically one H_2O blank, one liver blank, and seven liver standards are prepared during the extraction procedure. See FACT-M-1.

9.0 Sample Handling

9.1 Fresh liver standards are prepared with each analysis. Extracted standards and samples are stored in capped autovials or capped 15 mL centrifuge tubes until analysis.

9.2 If analysis will be delayed, extracted standards and samples may be refrigerated until analysis can be performed.

10.0 Quality Control

10.1 Matrix Blanks and Method Blanks

10.1.1 Analyze a method blank and matrix blank prior to each calibration curve.

10.2 Matrix Spikes

10.2.1 Analyze a matrix spike and matrix spike duplicate with each analysis.

10.2.2 Expected concentrations will fall in the mid-range of the initial calibration curve. Additional spike concentrations may fall in the low-range of the initial calibration curve.

10.2.3 See section 13 to calculate percent recovery.

10.3 Continuing Calibration Checks

10.3.1 Analyze a mid-range calibration standard after every tenth sample. If a significant change (± 30%) in peak area occurs, relative to the initial standard curve, stop the run. Only those samples analyzed before the last acceptable calibration standard will be used. The remaining samples must be reanalyzed.

10.3.2 See section 13 to calculate percent difference.

10.4 System Suitability

10.4.1 System suitability (e.g. peak area, retention time and peak shape, etc.) will be assessed for each run.

11.0 Calibration and Standardization

11.1 Analyze the extracted liver standards prior to and following each set of extracts. The mean of two standard values, at each standard concentration, will be plotted by linear regression for the calibration curve using MassLynx or other suitable software.

11.2 The r^2 value for the data should be 0.98 or greater. Lower values may be acceptable at the discretion of the analyst.

11.3 If the curve does not meet requirements, perform routine maintenance or reextract the standard curve (if necessary) and reanalyze.

12.0 PROCEDURES

12.1 Acquisition Set up

12.1.1 Click on start button in the Acquisition Control Panel. Set up a sample list. Assign a filename using letter-MO-DAY-last digit of year-sample number, assign a method (MS) for acquiring, and type in sample descriptions.

12.1.2 To create a method click on scan button in the Acquisition control panel and select SIR. Set Ionization Mode as appropriate and mass to 499 or other appropriate masses. A scan is usually collected along with the SIRs. Save method.

12.1.3 Typically the sample list begins with the first set of liver standards and ends with the second set of standards.

12.1.4 Samples are analyzed with a continuing calibration check injected after every tenth sample. Solvent blanks should be analyzed periodically to monitor possible analyte carryover and are not considered samples but may be included as such.

12.2 Using the Autosampler

12.2.1 Set up sample tray according to the sample list prepared in section 12.1.1.

12.2.2 Set-up the HP1100/autosampler at the following conditions or at conditions the analyst considers appropriate for optimal response. Record actual conditions in the instrument logbook:

12.2.2.1 Sample size = 10 µL injection with a sample wash

12.2.2.2 Inject/sample = 1

12.2.2.3 Cycle time = 15 minutes

12.2.2.4 Solvent ramp =

Time	MeOH	2.0 mM Ammonium acetate
0.00 min.	45%	55%
7.5 min.	90%	10%
11.0 min.	90%	10%
11.5 min.	45%	55%

Note: In this instrument configuration, the run must be set up on the electrospray software with a "Waiting for inlet start" message before the "Start" button is pressed on the HP Workstation.

12.2.2.5 Press the "Start" button.

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12.3 Instrument Sep-up

12.3.1 Refer to AMDT-EP-31 for more details.

12.3.2 Check the solvent level in reservoirs and refill if necessary.

12.3.3 Check the stainless steel capillary at the end of the probe. Use an eye piece to check the tip. The tip should be flat with no jagged edges. If the tip is found to be unsatisfactory, disassemble the probe and replace the stainless steel capillary.

12.3.4 Set HPLC pump to "On". Set the flow to 10 - 500 uL/min or as appropriate. Observe droplets coming out of the tip of the probe. Allow to equilibrate for approximately 10 minutes.

12.3.5 Turn on the nitrogen. A fine mist should be expelled with no nitrogen leaking around the tip of the probe.

12.3.6 The instrument uses these parameters at the following settings. These settings may change in order to optimize the response:

12.3.6.1 Drying gas 250-400 liters/hour

12.3.6.2 ESI nebulizing gas 10-15 liters/hour

12.3.6.3 LC constant flow mode flow rate 10 – 500 uL/min

12.3.6.4 Pressure <400 bar (This parameter is not set, it is a guide to ensure the instrument is operating correctly.)

12.3.7 Carefully guide the probe into the opening. Insert probe until it will not go any further. Connect the voltage cables to the probe.

12.3.8 Record tune parameters in the instrument log.

12.3.9 Using the cross-flow counter electrode in the ES/MS source is recommended for the analysis of biological matrices.

12.3.10 Click on start button in the Acquisition Control Panel. Press the start button at top of sample list. Ensure start and end sample number includes all samples to be analyzed.

13.0 DATA ANALYSIS AND CALCULATIONS

13.1 Calculations:

13.1.1 Calculate matrix spike percent recoveries using the following equation:

$$\% \text{ Recovery} = \frac{\text{Observed Result - Background Result}}{\text{Expected Result}} \times 100$$

13.1.2 Calculate percent difference using the following equation:

$$\% \text{ Difference} = \frac{\text{Expected Conc. - Calculated Conc.}}{\text{Expected Conc.}} \times 100$$

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13.1.3 Calculate actual concentration of PFOS anion in total liver (mg):

$$\frac{\left(\frac{\text{ug PFOS anion calc. from std curve}}{\text{g of liver used for analysis}}\right)}{1000\ \text{ug}/1\ \text{mg}} \times \text{Total mass of liver (g)}$$

14.0 Method Performance

14.1 The method detection limit is equal to at least three times the baseline noise in the matrix blank.

14.2 The practical quantitation limit is equal to the lowest standard in the calibration curve.

15.0 Pollution Prevention and Waste Management

15.1 Sample waste is disposed in biohazard containers, flammable solvent waste is disposed in high BTU containers, and glass pipette waste is disposed in broken glass containers. All containers are located in the laboratory.

16.0 Records

16.1 Store chromatograms in the study folder. Each chromatogram should have the following information included either in the header or hand written on the chromatogram: study number, sample name, extraction date, and dilution factor (if applicable).

16.2 Plot calibration curve by linear regression and store in the study folder.

16.3 Print sample list from MassLynx and tape into the instrument runlog.

16.4 Print data integration summary from MassLynx and tape into the instrument runlog.

16.5 Copy instrument runlog pages, including instrument parameters and sample results, and tape into appropriate study notebook.

16.6 Summarize data using suitable software and store in the study folder.

16.7 Back up electronic data to appropriate media. Record in study notebook the file name and location of backup electronic data.

17.0 Tables, Diagrams, Flowcharts, and Validation Data

17.1 Attachment A: FACT-M-2 Data reporting spreadsheet

17.2 The validation report associated with this method is FACT-M-1.0 & 2.0-V-1.

18.0 References

18.1 AMDT-EP-31, "Operation of VG Platform Electrospray Mass Spectrometer"

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19.0 AFFECTED DOCUMENTS

19.1 FACT-M-1.0, "Extraction of Potassium Perfluorooctanesulfonate from Liver for Analysis Using HPLC-Electrospray/Mass Spectrometry"

20.0 REVISIONS

Revision Number.	Reason For Revision	Revision Date

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Laboratory Study

Study:
Test Material:
Matrix/Final Solvent:
Method/Revision:
Analytical Equipment System Number:
Instrument Software/Version:
Filename:
R-Squared Value:
Slope:
Y Intercept:
Date of Extraction/Analyst:
Date of Analysis/Analyst:

Group Dose	Sample#	Concentration ug/mL	Initial Vol. mL	Dilution Factor	Final Conc. ug/mL

Slope: Taken from linear regression equation.
Group/Dose: Taken from the study folder.
Sample#: Taken from the study folder.
Concentration (ug/mL): Taken from the MassLynx integration summary.
Initial Volume (mL): Taken from the study folder.
Dilution Factor: Taken from the study folder.
Final Conc. (ug/mL): Calculated by dividing the initial volume from the concentration

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3M Environmental Laboratory

Method

Extraction of Potassium Perfluorooctanesulfonate or other Anionic Fluorochemical surfactants from Serum for Analysis Using HPLC-Electrospray/Mass Spectrometry

Method Number: FACT-M-3.0

Adoption Date: 4/22/98

Revision Date: N/A

Author: Lisa Clemen

Approved By:

Laboratory Manager — Date: 4/22/98

Group Leader — Date: 4/22/98

Technical Reviewer — Date: 4/22/98

1.0 Scope and Application

1.1 Scope: This method is for the extraction of potassium perfluorooctanesulfonate (PFOS) or other fluorochemical surfactants from serum.

1.2 Applicable Compounds: Fluorochemical surfactants or other fluorinated compounds.

1.3 Matrices: Rabbit, rat, and bovine serum or other sera as designated in the validation report.

2.0 SUMMARY OF METHOD

2.1 This method describes how to extract potassium perfluorooctanesulfonate (PFOS) or other anionic fluorochemical surfactants from serum using an ion pairing reagent and 5.0 mL of ethyl acetate. An ion pairing reagent is added to the sample and the analyte ion pair is partitioned into ethyl acetate. Four mL of extract are removed and put onto a nitrogen evaporator until dry. Each extract is reconstituted in 1.0 mL of methanol, then filtered through a 3 cc plastic syringe attached to a 0.2 µm nylon filter into glass autovials.

3.0 DEFINITIONS

3.1 None.

4.0 WARNINGS AND CAUTIONS

4.1 **Health and Safety Warnings:**

4.1.1 Use universal precautions, especially laboratory coats, goggles, and gloves when handling animal serum, it may contain pathogens.

5.0 INTERFERENCES

5.1 There are no known interferences at this time.

6.0 EQUIPMENT

6.1 The following equipment is used while carrying out this method. Equivalent equipment is acceptable.

6.1.1 Vortex mixer, VWR, Vortex Genie 2

6.1.2 Centrifuge, Mistral 1000 or IEC

6.1.3 Shaker, Eberbach or VWR

6.1.4 Nitrogen evaporator, Organomation

6.1.5 Balance, (± 0.100 gm)

7.0 SUPPLIES AND MATERIALS

7.1 Gloves

7.2 Eppendorf or disposable pipettes

7.3 Nalgene bottles, capable of holding 250 mL and 1 L

7.4 Glass, type A, volumetric flasks

7.5 40 mL glass I-CHEM vials

7.6 Polypropylene centrifuge tubes, 15 mL

7.7 Labels

7.8 Syringes, capable of measuring 10 µL to 50 µL

7.9 Glass, type A, volumetric pipettes

7.10 Graduated pipettes

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7.11 Electronic pipettor, Eppendorf or equivalent

7.12 Timer

7.13 Disposable plastic 3 cc syringes

7.14 Filters, nylon syringe filters, 0.2 µm, 25 mm

7.15 Crimp cap autovials

Note: Prior to using glassware and bottles, rinse 3 times with methanol and 3 times with Milli-Q™ water. Rinse syringes a minimum of 9 times with methanol, 3 rinses from 3 separate vials.

8.0 Reagents and Standards

8.1 Reagents

8.1.1 Sodium hydroxide (J.T Baker or equivalent), (NaOH) 10N: weigh approximately 200 grams NaOH. Pour into a 1000 mL beaker containing 500 liters (L) Milli-Q™ water, mix until all solids are dissolved. Store in a 1 L Nalgene bottle.

8.1.2 Sodium hydroxide (J.T Baker or equivalent), (NaOH) 1N. Dilute 10N 1:10. Measure 10 mL of 10N NaOH solution into a 100 mL volumetric flask and dilute to volume using Milli-Q™ water. Store in a 125 mL Nalgene bottle.

8.1.3 Tetrabutylammonium hydrogen sulfate (Kodak or equivalent), (TBA) 0.5M: Weigh approximately 169 grams of TBA into a 1 L volumetric containing 500 L Milli-Q™ water. Adjust to pH 10 using approximately 64 mL of 10N NaOH and dilute to volume with Milli-Q™ water. Add NaOH slowly while adding the last mL of NaOH because the pH changes abruptly. Store in a 1 L Nalgene bottle.

8.1.3.1 TBA requires a check prior to each use to ensure pH = 10. Adjust as needed using 1N NaOH solution.

8.1.4 Sodium carbonate/sodium bicarbonate buffer (J.T. Baker or equivalent), (Na_2CO_3/$NaHCO_3$) 0.25M: Weigh approximately 26.5 g of sodium carbonate (Na_2CO_3) and 21.0 g of sodium bicarbonate ($NaHCO_3$) into a 1 L volumetric flask and bring to volume with Milli-Q™ water. Store in a 1 L nalgene bottle.

8.1.5 PFOS (3M Specialty Chemical Division), molecular weight = 538.

8.1.6 Other fluorochemicals, as appropriate.

8.1.7 Ethyl Acetate, Omnisolv, glass distilled or HPLC grade.

8.1.8 Methanol, Omnisolv, glass distilled or HPLC grade.

8.1.9 Serum, frozen liquid from Sigma.

8.1.10 Control serum received with each sample set.

8.1.11 Milli-Q™ water, all water used in this method should be Milli-Q™ water and may be provided by a Milli-Q TOC Plus system.

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8.2 Standards

8.2.1 Prepare PFOS standards for the standard curve.

8.2.2 Prepare other fluorochemical standards, as appropriate.

8.2.3 Weigh approximately 100 mg of PFOS into a 100 mL volumetric flask and record the actual weight.

8.2.4 Bring to volume with methanol for a stock standard of approximately 1000 ppm (µg/mL).

8.2.5 Dilute the stock solution with methanol for a working standard 1 solution of approximately 50 ppm.

8.2.6 Dilute the stock solution with methanol for a working standard 2 solution of approx. 5.0 ppm.

8.2.7 Dilute the stock solution with methanol for a working standard 3 solution of approx. 0.50 ppm.

9.0 Sample Handling

9.1 All sera are received frozen and must be kept frozen until the extraction is performed.

10.0 Quality Control

10.1 Matrix Blanks and Method Blanks

10.1.1 Two 1.0 mL aliquots of the serum are extracted following this procedure and used as matrix blanks. See section 11.1.2.

10.1.2 Two 1.0 mL aliquots of Milli-Q™ water are extracted following this procedure and used as method blanks.

10.2 Matrix Spikes

10.2.1 Prepare and analyze matrix spike and matrix spike duplicate samples to determine the accuracy of the extraction.

10.2.2 Prepare each spike using serum chosen by the analyst, usually control serum received with each sample set.

10.2.3 Expected concentrations will fall in the mid-range of the initial calibration curve. Additional spikes may be included and may fall in the low-range of the initial calibration curve.

10.3 Continuing Calibration Checks

10.3.1 Prepare and analyze continuing calibration check samples to determine the continued linearity of the initial calibration curve.

10.3.2 One check is prepared per group of ten samples. For example, if a sample set = 34, four checks are prepared and extracted.

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10.3.3 Prepare each continuing calibration check from the same serum used to prep the initial curve.

10.3.4 The expected concentration will fall within the mid-range of the initial calibration curve.

11.0 CALIBRATION AND STANDARDIZATION

11.1 Prepare Serum Standards

11.1.1 Transfer 1 mL of serum to a 15 mL centrifuge tube.

11.1.2 If the majority of serum sample volumes are less than 1.0 mL, extract standards using serum volumes in the standards equal to the serum volumes in samples. Do not extract below 0.50 mL of serum. Record the serum volume on the extraction sheet.

11.1.3 Mix or shake between aliquots while preparing a total of sixteen aliquots of serum in 15 mL centrifuge tubes.

11.1.4 Two 1 mL or appropriate aliquots serve as matrix blanks. Typically use the standard concentrations and spiking amounts listed in table 1 to spike, in duplicate, two standard curves for a total of fourteen samples.

11.1.5 Refer to the validation report FACT-M-3.0-V-1 and FACT-M-4.0-V-1 which lists the working ranges for calibration curves.

Table 1
Approximate Spiking Amounts for Standards and Spikes Using 1.0 mL of Serum

Working Standard (Approx. Conc.)	µL	Approx. final conc. of PFOS in serum
-	-	Blank
0.500 ppm	20	0.010 ppm
5.00 ppm	5	0.025 ppm
5.00 ppm	10	0.050 ppm
5.00 ppm	20	0.100 ppm
50.0 ppm	5	0.250 ppm
50.0 ppm	10	0.500 ppm
50.0 ppm	15	0.750 ppm

11.1.4 See section 13.0 to calculate actual concentrations of PFOS in calibration standards.

11.2 Extract spiked serum standards following **12.6-12.16** of this method. Use these standards to establish each initial curve on the mass spectrometer.

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12.0 PROCEDURES

12.1 Obtain frozen serum samples and allow to thaw.

12.2 Vortex mix for 15 seconds then remove 1.0 mL or appropriate volume to a 15 mL polypropylene centrifuge tube.

12.3 Return serum samples to freezer after extraction amount has been removed.

12.4 Record the serum volume on the extraction worksheet. The final methanol volume will equal the initial serum volume.

12.5 Label the tube with the study number, serum ID, date and analyst initials. See attached worksheet for documenting the remaining steps.

12.6 Spike serum with the appropriate amount of PFOS standard as described in section 11.1 or Table I for the calibration curve standards. Also spike matrix spikes and continuing calibration standards.

12.7 Vortex mix the standard curve samples, matrix spike samples, and continuing calibration samples for 15 seconds.

12.8 Add 1 mL 0.5 M TBA and 2 mL of the 0.25 M sodium carbonate/sodium bicarbonate buffer.

12.9 Using a volumetric pipette, add 5 mL ethyl acetate.

12.10 Cap each sample and put on the shaker for 20 minutes.

12.11 Centrifuge for 20 to 25 minutes, until layers are well separated. Set power on the centrifuge to approximately 3500 rpm.

12.12 Transfer 4 mL of organic layer, using a 5 mL graduated glass pipette, to a clean 15 mL centrifuge tube. Label this fresh tube with the same information as in **12.5**.

12.13 Put each sample on the analytical nitrogen evaporator until dry, approximately 2 to 3 hours.

12.14 Add 1.0 mL or appropriate volume of methanol to each centrifuge tube using a graduated pipette. (This volume equals the initial volume of serum used for the extraction.)

12.15 Vortex mix for 30 seconds.

12.16 Attach a 0.2 µm nylon mesh filter to a 3 cc syringe and transfer the sample to this syringe. Filter into a 1.5 mL glass autovial.

12.17 Label the autovial with the study number, animal number and gender, sample timepoint, matrix, final solvent, extraction date, and analyst(s) who performed the extraction.

12.18 Cap and hold for HPLC-electrospray/mass spectrometry analysis. Extracts may be stored at 4° C until analysis.

12.19 Complete the extraction worksheet, attached to this document, and tape to page of study notebook.

Extraction Worksheet for FACT-M-3

Study #	Sample Number set #	PFOS approx. 0.5 ppm actual ppm #W_____	PFOS approx. 5 ppm actual ppm #W_____	PFOS approx. 50 ppm actual ppm #W_____	Date and Initials for Std. or Comments
-	H_2O Blank	-	-	-	
-	Serum Blank	-	-	-	
-					
-					
-					
-					
-					
-					
-					
[1]		-	-	-	
		-	-	-	
		-	-	-	
		-	-	-	
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		-	-	-	
		-	-	-	
		-	-	-	
		-	-	-	
		-	-	-	
		-	-	-	

[1] Study number where the original worksheet is located.

Blank Serum Std # Serum amount = g

Serum Extraction Method : **Date & Initials**

Vortex 15 sec.

Pipette Serum Volume mL

Pipette 1 mL of 0.5 M TBA, pH 10. Std. #

Pipette 2 mL of 0.25 Na_2CO_3/0.25M $NaHCO_3$ buffer Std. #

Pipette 5 mL of ethyl acetate TN-A-

Shake 20 min.

Centrifuge 20-25 min. Centrifuge speed:

Remove a 4 mL aliquot of organic laver

Put on Nitrogen Evaporator to dryness Evaporator #: Temperature:

Add methanol Volume mL TN-A-

Vortex 30 sec.

Filter using a 3cc B-D syringe with a 0.2µm SRI filter into a 1.5 mL autosample vial

MS/MSD/___ Cont. Checks: Spiked _____ uL of a _____ ppm std (_____) for a final concentration of _____ ppm. MS/MSD used sample _____. Cont. Checks used same serum as for std curve.

3M ENVIRONMENTAL LABORATORY

METHOD

ANALYSIS OF FLUOROCHEMICALS IN SERUM EXTRACTS USING HPLC-ELECTROSPRAY/MASS SPECTROMETRY

Method Number: FACT-M-4.0

Adoption Date: 4/22/98

Revision Date: N/A

Author: Lisa Clemen

Approved By:

Laboratory Manager — Date: 4/22/98

Group Leader — Date: 4/22/98

Technical Reviewer — Date: 4/14/98

1.0 SCOPE AND APPLICATION

1.1 Scope: This method is for the analysis of extracts of serum or tissue for fluorochemical surfactants using HPLC-electrospray/mass spectrometry.

1.2 Applicable Compounds: Potassium perfluorooctanesulfonate, anionic fluorochemical surfactants, or other ionizable compounds.

1.3 Matrices: Rabbit, rat, and bovine serum or other sera as designated in the validation report.

2.0 Summary of Method

2.1 This method describes the analysis of fluorochemical surfactants extracted from serum using HPLC-electrospray/mass spectrometry. The analysis is performed by monitoring a single ion characteristic of a particular fluorochemical, such as the potassium perfluorooctanesulfonate (PFOS) anion, M/Z= 499. Samples may also be screened to verify compound identification.

3.0 Definitions

3.1 None.

4.0 Warnings and Cautions

4.1 Health and Safety Warnings:

4.1.1 Use caution with the voltage cable for the probe. When the voltage cable is plugged into the probe DO NOT TOUCH THE PROBE, there is risk of electrical shock.

4.2 Cautions:

4.2.1 Do not run solvent pumps above capacity of 400 bar (5800 psi). If pressure goes over 400 bar, the HP1100 will initiate automatic shutdown.

4.2.2 Do not run solvent pumps to dryness.

5.0 Interferences

5.1 Teflon should not be used for sample storage or any part of instrumentation that comes in contact with the sample or extract.

6.0 Equipment

6.1 Equipment listed below may be changed in order to optimize the system.

6.1.1 Micromass Electrospray Mass Spectrometer

6.1.2 HP1100 low pulse solvent pumping system and autosampler.

7.0 Supplies and Materials

7.1 Supplies

7.1.1 Nitrogen gas, refrigerated liquid, regulated to approximately 100 psi.

7.1.2 HPLC column, specifics to be determined by the analyst.

7.1.3 Capped autovials or capped 15 mL centrifuge tubes.

8.0 Reagents and Standards

8.1 Reagents

8.1.1 Methanol, HPLC grade or equivalent.

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8.1.2 Milli-Q™ water, all water used in this method should be Milli-Q™ water and may be provided by a Milli-Q TOC Plus system.

8.1.3 Ammonium acetate, HPLC grade or equivalent.

8.2 Standards

8.2.1 Typically one H_2O blank, one serum blank, and seven serum standards are prepared during the extraction procedure. See FACT-M-3.

9.0 Sample Handling

9.1 Fresh serum standards are prepared with each analysis. Extracted standards and samples are stored in capped autovials or capped 15 mL centrifuge tubes until analysis.

9.2 If analysis will be delayed, extracted standards and samples may be refrigerated at 4° C until analysis can be performed.

10.0 Quality Control

10.1 Matrix Blanks and Method Blanks

10.1.1 Analyze a method blank and a matrix blank prior to each calibration curve.

10.2 Matrix Spikes

10.2.1 Analyze a matrix spike and matrix spike duplicate with each analysis.

10.2.2 Expected concentrations will fall in the mid-range of the initial calibration curve. Additional spike concentrations may fall in the low-range of the initial calibration curve.

10.2.3 See section 13 to calculate percent recovery.

10.3 Continuing Calibration Checks

10.3.1 Analyze a mid-range calibration standard after every tenth sample. If a significant change (± 30%) in peak area occurs, relative to the initial standard curve, stop the run. Only those samples analyzed before the last acceptable calibration standard will be used. The remaining samples must be reanalyzed.

10.3.2 See section 13 to calculate percent difference.

10.4 System Suitability

10.4.1 System suitability (e.g., peak area, retention time, peak shape, etc.) will be assessed for each run.

11.0 Calibration and Standardization

11.1 Analyze the extracted serum standards prior to and following each set of extracts. The mean of two standard values, at each standard concentration, will be plotted by linear regression for the calibration curve using MassLynx or other suitable software.

11.2 The r^2 value for the data should be 0.98 or greater. Lower values may be acceptable at the discretion of the analyst.

11.3 If the curve does not meet requirements, perform routine maintenance or reextract the standard curve (if necessary) and reanalyze.

12.0 PROCEDURES

12.1 Acquisition Set up

12.1.1 Click on start button in the Acquisition Control Panel. Set up a sample list. Assign a filename using letter-MO-DAY-last digit of year-sample number, assign a method (MS) for acquiring, and type in sample descriptions.

12.1.2 To create a method click on scan button in the Acquisition control panel and select SIR (Single Ion Recording). Set Ionization Mode as appropriate and mass to 499 or other appropriate masses. A scan is usually collected along with the SIRs. Save method.

12.1.3 Typically the sample list begins with the first set of serum standards and ends with the second set of standards.

12.1.4 Samples are analyzed with a continuing calibration check injected after every tenth sample. Solvent blanks should be analyzed periodically to monitor possible analyte carryover and are not considered samples but may be included as such.

12.2 Using the Autosampler

12.2.1 Set up sample tray according to the sample list prepared in section 12.1.1.

12.2.2 Set-up the HP1100/autosampler at the following conditions or at conditions the analyst considers appropriate for optimal response. Record actual conditions in the instrument logbook:

12.2.2.1 Sample size = 10 µL injection with a sample wash

12.2.2.2 Inject/sample = 1

12.2.2.3 Cycle time = 15 minutes

12.2.2.4 Solvent ramp =

Time	MeOH	2.0 mM Ammonium acetate
0.00 min.	45%	55%
7.5 min.	90%	10%
11.0 min.	90%	10%
11.5 min.	45%	55%

Note: In this instrument configuration, the run must be set up on the electrospray software with a "Waiting for inlet start" message before the "Start" button is pressed on the HP Workstation.

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12.2.2.5 Press the "Start" button.

12.3 Instrument Set-up

12.3.1 Refer to AMDT-EP-31 for more details.

12.3.2 Check the solvent level in reservoirs and refill if necessary.

12.3.3 Check the stainless steel capillary at the end of the probe. Use an eye piece to check the tip. The tip should be flat with no jagged edges. If the tip is found to be unsatisfactory, disassemble the probe and replace the stainless steel capillary.

12.3.4 Set HPLC pump to "On". Set the flow to 10 - 500 uL/min or as appropriate. Observe droplets coming out of the tip of the probe. Allow to equilibrate for approximately 10 minutes.

12.3.5 Turn on the nitrogen. A fine mist should be expelled with no nitrogen leaking around the tip of the probe.

12.3.6 The instrument uses these parameters at the following settings. These settings may change in order to optimize the response:

12.3.6.1 Drying gas 250-400 liters/hour

12.3.6.2 ESI nebulizing gas 10-15 liters/hour

12.3.6.3 HPLC constant flow mode flow rate 10 – 500 µL/min

12.3.6.4 Pressure <400 bar (This parameter is not set, it is a guide to ensure the HPLC is operating correctly.)

12.3.7 Carefully guide the probe into the opening. Insert probe until it will not go any further. Connect the voltage cables to the probe.

12.3.8 Record tune parameters in the instrument log.

12.3.9 Using the cross-flow counter electrode in the ES/MS source is recommended for the analysis of biological matrices.

12.3.10 Click on start button in the Acquisition Control Panel. Press the start button at top of sample list. Ensure start and end sample number includes all samples to be analyzed.

13.0 Data Analysis and Calculations

13.1 Calculations:

13.1.4 Calculate matrix spike percent recoveries using the following equation:

$$\% \text{ Recovery} = \frac{\text{Observed Result - Background Result}}{\text{Expected Result}} \times 100$$

13.1.5 Calculate percent difference using the following equation:

$$\% \text{ Difference} = \frac{\text{Expected Conc. - Calculated Conc.}}{\text{Expected Conc.}} \times 100$$

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13.1.6 Calculate actual concentration of PFOS, or other fluorochemical, anion in serum (µg/mL):

$$\frac{\text{µg of PFO calc. from std. Curve}}{\text{Initial Volume of serum (mL)}} \times \text{Dilution Factor} \times \text{Final Volume (mL)}$$

14.0 Method Performance

14.1 The method detection limit is equal to half the lowest standard in the calibration curve.

14.2 The practical quantitation limit is equal to the lowest standard in the calibration curve.

15.0 Pollution Prevention and Waste Management

15.1 Sample extract waste and flammable solvent is disposed in high BTU containers, and glass pipette waste is disposed in broken glass containers located in the laboratory.

16.0 Records

16.1 Store chromatograms in the study folder. Each chromatogram must have the following information included either in the header or hand written on the chromatogram: study number, sample name, extraction date, and dilution factor (if applicable).

16.2 Plot calibration curve by linear regression and store in the study folder.

16.3 Print sample list from MassLynx and tape into the instrument runlog.

16.4 Print data integration summary from MassLynx and tape into the instrument runlog.

16.5 Copy instrument runlog pages, including instrument parameters and sample results, and tape into appropriate study notebook.

16.6 Summarize data using suitable software and store in the study folder.

16.7 Back up electronic data to appropriate medium. Record in study notebook the file name and location of backup electronic data.

17.0 Tables, Diagrams, Flowcharts, and Validation Data

17.1 Attachment A: FACT-M-4 Data reporting spreadsheet

17.2 The validation report associated with this method is FACT-M-3.0 & 4.0-V-1.

18.0 References

18.1 AMDT-EP-31, "Operation of VG Platform Electrospray Mass Spectrometer"

19.0 Affected Documents

19.1 FACT-M-3.0, "Extraction of Fluorochemical Anions from Serum for Analysis Using HPLC-Electrospray/Mass Spectrometry"

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20.0 REVISIONS

Revision Number.	Reason For Revision	Revision Date

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Laboratory Study

Study:
Test Material:
Matrix/Final Solvent:
Method/Revision:
Analytical Equipment System Number:
Instrument Software/Version:
Filename:
R-Squared Value:
Slope:
Y Intercept:
Date of Extraction/Analyst:
Date of Analysis/Analyst:

Group Dose	Sample#	Concentration ug/mL	Initial Vol. mL	Dilution Factor	Final Conc. ug/mL

Slope: Taken from linear regression equation.
Group/Dose: Taken from the study folder.
Sample#: Taken from the study folder.
Concentration (ug/mL): Taken from the MassLynx integration summary.
Initial Volume (mL): Taken from the study folder.
Dilution Factor: Taken from the study folder.
Final Conc. (ug/mL): Calculated by dividing the initial volume from the concentration

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ATTACHMENT G:
RESULTS OF CONFIRMATORY DOSE ANALYSES

Study: Argus 418-008, Two-Generation Reproduction Study of PFOS in Rats
Product Number(Test Substance): PFOS (T-6295.9)
Matrix: Tween Dosing Vehicle
Method/Revision: ETS-8-4.1 & ETS-8-5.1
Analytical Equipment System Number: Madeline041098
Instrument Software/Version: MassLynx 3.2
Date of Extraction/Analyst: 9/13/99 IAS, 9/23/99 IAS
Date of Analysis/Analyst: 9/15/99 IAS, 9/24/99 IAS
Date of Data Reduction/Analyst: 9/16/99 IAS, 9/27/99 IAS

Filename: See list to right
R-Squared Value: See Attachments
Slope: See Attachments
Y-Intercept: See Attachments

Filenames

	PFOS
Grp 1	091599013
Grp 2	091599014
Grp 3	091599015
Grp 4	091599016
Grp 5	092799149,152
MS, MSD	091599020-023, 092799159-160

Sample Data

Tween Dosing Confirmation

Group Dose	Sample #	Expected Conc. PFOS ng/mL	PFOS Conc. ng/mL	Dilution Factor	Total PFOS ng/mL	PFOS-Bckgrnd Conc. ng/mL	PFOS % Recovery Accuracy
Method Blk	H2O Blk-1	NA	NA	NA	NA	NA	NA
Method Blk	H2O Blk-2	NA	NA	NA	NA	NA	NA
Matrix Blk	Rabbit Liver Blk-1	NA	NA	NA	NA	NA	NA
Matrix Blk	Rabbit Liver Blk-2	NA	NA	NA	NA	NA	NA
QC	B-418-008-B (302ppb MS, ext9/13/99)	302	778	NA	NA	266	88%
	B-418-008-C (50.3ppb MS, ext9/13/99)	50.3	130	NA	NA	60	119%
	B-418-008-D (151ppb MS, ext9/13/99)	151	461	NA	NA	159	105%
	B-418-008-E (302ppb MS, ext9/13/99)	302	852	NA	NA	288	95%
	B-418-008-E (302ppb MS ext9/23/99)	302	865	NA	NA	284	94%
Group 1 Control 0.0 mg/kg/day 0 ng/mL	B-418-008-A,12/27/98 (ext 9/13/99) Diluted 1/1	0.00	0.0	1	0.0	NA	<LOD
Group 2 0.1 mg/kg/day 0.02 ng/mL	B-418-008-B,12/21/98 (ext 9/13/99) Diluted 1/40	20000	512	40	20489	NA	102%
Group 3 0.4 mg/kg/day 0.08 ng/mL	B-418-008-C,12/21/98 (ext 9/13/99) Diluted 1/1000	80000	70.1	1000	70050	NA	88%
Group 4 1.6 mg/kg/day 032 ng/mL	B-418-008-D,12/21/98 (ext 9/13/99) Diluted 1/1000	320000	303	1000	302550	NA	95%
Group 5 3.2 mg/kg/day 0.64 ng/mL	B-418-008-E,12/21/98 (ext 9/13/99)	640000	565	1000	564560	NA	88%
	B-418-008-E,12/21/98 (ext 9/23/99) Diluted 1/1000	640000	581	1000	580860	NA	91%

Limit of Quantitation Limit (LOQ) = PFOS = 30 ng/g
Method Detection Limit (MDL): PFOS = 15 ng/g
PFOS = Perfluorooctanesulfonate

NR = Sample not received nor reported.
NA = Not Applicable

Date Entered/Analyst: 9/27/99 GML
Date Verified/Analyst:

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Study:
Product Number(Test Substance):
Matrix:
Method/Revision:
Analytical Equipment System Number:
Instrument Software/Version:
Date of Extraction/Analyst:
Date of Analysis/Analyst:
Date of Data Reduction/Analyst:

Argus 418-008, Two-Generation Reproduction Study of PFOS in Rats
PFOS (T-6295.9)
Tween Dosing Vehicle
ETS-8-4.1 & ETS-8-5.1
Madeline041098
MassLynx 3.2
9/13/99 IAS, 9/23/99 IAS
9/15/99 IAS, 9/24/99 IAS
9/16/99 IAS, 9/27/99 IAS

Filename: See Attachments
R-Squared Value: See Attachments
Slope: See Attachments
Y-Intercept: See Attachments

Sample Data

Tween Dosing Confirmation

Group Dose 0	Sample # 0	Expected Conc. PFOS ng/mL	PFOS Conc. ng/mL	PFOS % Recovery Accuracy
Method Blk	H2O Blk-1	NA	NA	NA
Method Blk	H2O Blk-2	NA	NA	NA
Matrix Blk	Rabbit Liver Blk-1	NA	NA	NA
Matrix Blk	Rabbit Liver Blk-2	NA	NA	NA
QC	B-418-008-B (302ppb MS, ext9/13/99)	302	266	88%
	B-418-008-C (50 3ppb MS, ext9/13/99)	50	60	119%
	B-418-008-D (151ppb MS, ext9/13/99)	151	159	105%
	B-418-008-E (302ppb MS, ext9/13/99)	302	288	95%
	B-418-008-E (302ppb MS ext9/23/99)	302	284	94%
Group 1 Control 0.0 mg/kg/day	B-418-008-A,12/27/98 (ext 9/13/99) Diluted 1/1	0.00	0.00	<LOD
Group 2 0.1 mg/kg/day 0.02 mg/mL	B-418-008-B,12/21/98 (ext 9/13/99) Diluted 1/40	20000	20489	102%
Group 3 0.4 mg/kg/day 0.08 mg/mL	B-418-008-C,12/21/98 (ext 9/13/99) Diluted 1/1000	80000	70050	88%
Group 4 1.6 mg/kg/day 032 mg/mL	B-418-008-D,12/21/98 (ext 9/13/99) Diluted 1/1000	320000	302550	95%
Group 5 3.2 mg/kg/day 0.64 mg/mL	B-418-008-E,12/21/98 (ext 9/13/99)	640000	564560	88%
	B-418-008-E,12/21/98 (ext 9/23/99)	640000	580860	91%

Limit of Quantitation Limit (LOQ) = PFOS = 30 ng/g
Method Detection Limit (MDL): PFOS = 15 ng/g
PFOS = Perfluorooctanesulfonate

NR = Sample not received nor reported.
NA = Not Applicable

Date Entered/Analyst: 9/27/99 GML
Date Verified/Analyst: 0

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3M SPECIALTY ADHESIVES & CHEMICALS ANALYTICAL LABORATORY

Request #'s 53030

To: Leo Gehlhoff - (309727) -3M Chemicals - 236-2A-01

From: Tom Kestner - (3-5633) SA&C Analytical Lab - 236-2B-11

Subject: ***Fluorochemical Isomer Distribution by ^{19}F-NMR Spectroscopy***

Date: December 1, 1997

SAMPLE DESCRIPTIONS:

- FC-95, lot # 217 (T-6295); Nominal product = C_8F_{17}-SO_3(-) K(+)

INTRODUCTION:

This sample was subjected to a ^{19}F-NMR spectral analysis method to determine the identities and relative concentrations of the fluorochemical isomers and as many other identifiable impurity components as possible.

EXPERIMENTAL:

A portion of the sample solid was totally dissolved in DMSO-d_6 and then a 376 MHz ^{19}F-NMR spectrum (F53030.401) was acquired using a Varian UNITYplus 400 FT-NMR spectrometer. Aida Robbins prepared the sample for analysis and she also acquired and plotted the NMR spectrum.

RESULTS:

The ^{19}F-NMR spectrum was used to determine the identities and relative concentrations of the nominal fluorochemical isomers and three other impurity components in this sample. The qualitative and quantitative compositional results which were derived from the single trial ^{19}F-NMR spectral analysis are summarized in **TABLE-1** on the following page.

A copy of the NMR spectrum and the spectral assignments data page are attached for your reference. If you have any questions about these NMR results, please let me know. I apologize for the delay in completing this work.

Tom Kestner

c: Jim Johnson - EE&PC - 2-3E-09

File Reference: LG53030.DOC/43

TABLE-1
Sample: T-6295 (FC-95, Lot 217)
^{19}F-NMR Compositional Results

Structural Assignments	^{19}F-NMR Relative Mole% Concentrations
$CF_3(CF_2)_x$-SO_3(-) K(+) (Normal chain, where x is mainly 7)	70.0%
$CF_3(CF_2)_x$-$CF(CF_3)$-$(CF_2)_y$- SO_3(-) K(+) (Internal monomethyl branch, where x+y is mainly 5, and $x \neq 0$, $y \neq 0$)	17.0%
$(CF_3)_2CF$-$(CF_2)_x$- SO_3(-) K(+) (Isopropyl branch, where x is mainly 5)	10.3%
C_xF_{2x+1}-$CF(CF_3)$- SO_3(-) K(+) (Alpha branch, where x is mainly 6)	1.6%
R_f-CF_2-SF_4-F	0.35%
$(CF_3)_3C$-$(CF_2)_x$- SO_3(-) K(+) (t-butyl branch, where x is mainly 4)	0.23%
CF_3-$(CF_2)_x$-$C(CF_3)_2$-$(CF_2)_y$- SO_3(-) K(+) (Internal gem-dimethyl branch, where x+y is mainly 4, and $x \neq 0$)	0.15%
Possible CF_3-SO_3(-) K(+)	0.25%
Possible CF_3-CO_2(-) K(+)	0.05%